

## AGONIST ANTIBODIES

This application is a non-provisional application filed under 37 CFR 1.53(b), claiming priority under 35 USC 119(e) to provisional application number 60/056,736, filed 22 August 1997, the contents of which are  
5 incorporated herein by reference.

Field of the Invention

This invention relates to the recombinant synthesis and purification of protein antibodies that influence survival, proliferation, differentiation or maturation of hematopoietic cells, especially platelet progenitor cells and to antibodies that influence the growth and differentiation of cells expressing a protein kinase receptor.  
10 This invention also relates to the cloning and expression of nucleic acids encoding antibody ligands (thrombopoietin receptor agonist antibodies) capable of binding to and activating a thrombopoietin receptor such as c-*mpl*, a member of the cytokine receptor superfamily. This invention further relates to the use of these antibodies alone or in combination with other cytokines to treat immune or hematopoietic disorders including thrombocytopenia and to uses in assays.

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Background of the Invention

In 1994 several groups reported the isolation and cloning of thrombopoietin (F. de Sauvage *et al.*, *Nature* 369:533 (1994); S. Lok *et al.*, *Nature* 369:565 (1994); TD. Bartley *et al.*, *Cell* 77:1117 (1994); Y. Sohma *et al.*, *FEBS Letters* 353:57 (1994); DJ. Kuter *et al.*, *Proc. Natl. Acad. Sci.* 91:11104 (1994)). This was the culmination of more than 30 years of research initiated in the late 50's when Yamamoto (S. Yamamoto, *Acta Haematol Jpn.* 20:163-178. (1957)) and Kelemen (E. Kelemen *et al.*, *Acta Haematol* (Basel). 20:350-355 (1958)) proposed that physiological platelet production is controlled by a humoral factor termed "thrombopoietin"(TPO). Although routinely detected in urine, plasma and serum from thrombocytopenic animals and patients, as well as kidney cell conditioned media, purification of TPO proved to be a daunting task (for a review see MS. Gordon *et al.*, *Blood* 80:302 (1992); W. Vainchenker *et al.*, *Critical Rev. Oncology/Hematology* 20:165 (1995)). In the absence of purified TPO and the apparent fact that numerous pleiotrophic cytokines affected megakaryocytopoiesis (MS. Gordon *et al.*, *Blood* 80:302 (1992); W. Vainchenker *et al.*, *Critical Rev. Oncology/Hematology* 20:165 (1995)), the existence of a lineage specific factor that regulated platelet production was doubted until the discovery of the orphan cytokine receptor c-Mpl in 1990 (M. Souyri *et al.*, *Cell* 63:1137 (1990); I. Vigon *et al.*, *Proc. Natl. Acad. Sci.* 89:5640 (1992)). The expression of c-Mpl was found to be restricted to progenitor cells, megakaryocytes and platelets, and c-Mpl antisense oligonucleotides selectively inhibited *in vitro* megakaryocytopoiesis (M. Methia *et al.*, *Blood* 82:1395 (1993)). From this it was postulated that c-Mpl played a critical role in regulating megakaryocytopoiesis and that its putative ligand may be the long sought TPO (M. Methia *et al.*, *supra*). Following this discovery several groups utilizing c-Mpl ligand specific cell proliferation assays and c-Mpl as a purification tool isolated and 30 cloned the ligand for c-Mpl (F. de Sauvage *et al.*, *supra*; S. Lok *et al.*, *supra*; TD. Bartley *et al.*, *supra*). In addition two other groups independently reported the purification of the Mpl-ligand using standard chromatography techniques and megakaryocyte assays (Y. Sohma *et al.*, *supra*; DJ. Kuter *et al.*, *supra*). In the years since its reported discovery numerous studies clearly indicate that the Mpl-ligand possess all the characteristics that have long been attributed to the purported regulator of megakaryocytopoiesis and  
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thrombopoiesis and consequently, is now referred to as TPO. The Mpl ligand is currently referred to as either TPO or as megakaryocyte growth and differentiation factor (MGDF).

Human TPO consists of 332 amino acids that can be divided into 2 domains; an amino terminal domain of 153 amino acids showing 23% identity (50% similarity) to erythropoietin (EPO) and a unique 181 amino acid C-terminal domain that is highly glycosylated ((F. de Sauvage *et al.*, *supra*; S. Lok *et al.*, *supra*; TD. Bartley *et al.*, *supra*). The EPO-like domain of TPO contains 4 cysteines, 3 of which are conserved with EPO. The first and last and the two middle cysteines form two disulfide bridges, respectively, which are both required for activity (T. Kato *et al.*, *Blood* 86 (suppl 1):365 (1995)). None of the Asn-linked glycosylation sites present in EPO are conserved in the EPO-like domain of TPO, however, the EPO-like domain of recombinant TPO (rTPO) contains 2-3 O-linked glycosylations (M. Eng *et al.*, *Protein Science* 5(suppl 1):105 (1996)). A recombinant truncated form of TPO (rTPO153), consisting of only the EPO-like domain, is fully functional *in vitro*, indicating that this domain contains all the required structural elements to bind and activate Mpl (F. de Sauvage *et al.*, *supra*; DL. Eaton *et al.*, *Blood* 84(suppl 1):241 (1994)). The carboxy terminal domain of TPO contains 6 N-linked and 18 O-linked glycosylated sites and is rich in proline, serine and threonine (M. Eng *et al.*, *supra*). The function of this domain remains to be elucidated. However, because of its high degree of glycosylation this region may act to stabilize and increase the half life of circulating TPO. This is supported by the observation that rTPO153 has a half life of 1.5 hours compared to 18-24 hours for full length glycosylated rTPO (GR. Thomas *et al.*, *Stem Cells* 14(suppl 1) (1996)).

The two domains of TPO are separated by a potential dibasic proteolytic cleavage site that is conserved among the various species examined. Processing at this site could be responsible for releasing the C-terminal region from the EPO domain *in vivo*. The physiological relevance of this potential cleavage site is unclear at this time. Whether TPO circulates as an intact full length molecule or as a truncated form is also equivocal. When aplastic porcine plasma was subjected to gel filtration chromatography, TPO activity present in this plasma resolved with a Mr. of ~150,000 ((F. de Sauvage *et al.*, *supra*). Purified full length rTPO also resolves at this Mr., whereas the truncated forms resolve with Mr. ranging from 18,000-30,000. Using TPO ELISAs that selectively detect either full length or truncated TPO it has also been shown that full length TPO is the predominant form in the plasma of marrow transplant patients (YG. Meng *et al.*, *Blood* 86(suppl. 1):313 (1995)).

Prior to the discovery of c-Mpl and the isolation of TPO, it was thought that megakaryocytopoiesis was regulated at multiple cellular levels (MS. Gordon *et al.*, *supra*; W. Vainchenker *et al.*, *supra*; YG. Meng *et al.*, *supra*). This hypothesis was based on the observation that certain hematopoietic growth factors stimulated proliferation of megakaryocyte progenitors while others primarily affected maturation (MS. Gordon *et al.*, *supra*; W. Vainchenker *et al.*, *supra*; YG. Meng *et al.*, *supra*). Other data indicated that plasma from thrombocytopenic animals contained distinct activities that either affected proliferation (meg-CSF) or maturation (TPO) of megakaryocytes (RJ. Hill *et al.*, *Exp. Hematol* 20:354 (1992)). Wendling and her colleagues (F. Wendling *et al.*, *Nature* 369:571 (1994)) initially dispelled this theory by demonstrating that all the megakaryocyte colony-stimulating and thrombopoietic activities in thrombocytopenic plasma could be neutralized by soluble Mpl. This indicated that these activities are due to a single factor, the Mpl-ligand. Numerous studies have now shown that recombinant forms of TPO not only induce proliferation of progenitor

megakaryocytes but also their maturation ( K. Kaushansky *et al.*, *Nature* 369:568 (1994); FC. Zeigler *et al.*, *Blood* 84:4045 (1994); VC. Broudy *et al.*, *Blood* 85:1719 (1995); JL. Nichol *et al.*, *J. Clin. Invest.* 95:2973 (1995); N. Banu *et al.*, *Blood* 86:1331 (1995); N. Debili *et al.*, *Blood* 86:2516 (1995); P. Angchaisuksiri *et al.*, *Br. J. Haematol.* 93:13 (1996); ES. Choi *et al.*, *Blood* 85:402 (1995)). Human CD34+, CD34+CD41+ cells (FC.

5 Zeigler *et al.*, *supra*; VC. Broudy *et al.*, *supra*; JL. Nichol *et al.*, *supra*; N. Banu *et al.*, *supra*;) or purified murine stem cells (sca+,lin-, kit+) (K. Kaushansky *et al.*, *supra*; FC. Zeigler *et al.*, *supra*) cultured with rTPO selectively differentiate to megakaryocytes. rTPO induces the differentiation and proliferation of megakaryocyte colonies in semisolid cultures and single megakaryocytes in liquid suspension cultures. This activity appears to be a direct effect of TPO as limiting dilution experiments show a direct correlation between 10 progenitors seeded and megakaryocytes obtained (N. Debili *et al.*, *supra*). In addition comparable results are obtained in serum free or serum containing culture conditions (N. Banu *et al.*, *supra*; N. Debili *et al.*, *supra*; P. Angchaisuksiri *et al.*, *supra*). These observations indicate that neither accessory cells or serum components are required for TPO to induce megakaryocyte growth and differentiation in vitro.

15 The effect of rTPO on the megakaryocyte maturation process is dramatic. rTPO induces highly purified murine or human progenitor cells in liquid culture to differentiate into very large mature polyploid megakaryocytes (FC. Zeigler *et al.*, *supra*; VC. Broudy *et al.*, *supra*; JL. Nichol *et al.*, *supra*; N. Debili *et al.*, *supra*). Megakaryocytes from such cultures exhibit ploidy of 4N-16N with ploidy classes of 64N and 128N also being detected in these cultures (N. Debili *et al.*, *supra*). In addition, megakaryocytes produced from these 20 cultures undergo a terminal maturation process and appear to develop proplatelets and shed platelet like structures into the medium (FC. Zeigler *et al.*, *supra*; N. Debili *et al.*, *supra*; ES. Choi *et al.*, *supra*). Significantly, the platelets produced from such cultures have been shown to be morphologically and functionally indistinct from plasma-derived platelets (ES. Choi *et al.*, *supra*).

25 Although, rTPO appears to act directly on hematopoietic progenitors to induce megakaryocyte differentiation, it also acts synergistically and additively with early and late acting hematopoietic factors. In murine megakaryocytopoiesis assays IL-11, kit ligand (KL) or EPO act synergistically and IL-3 and IL-6 act additively with rTPO to stimulate proliferation of megakaryocyte progenitors (VC. Broudy *et al.*, *supra*). In human megakaryocytopoiesis assays IL-3 and IL-6 effects are additive to rTPO, while KL acts synergistically with rTPO (JL. Nichol *et al.*, *supra*; N. Banu *et al.*, *supra*; N. Debili *et al.*, *supra*; P. Angchaisuksiri *et al.*, *supra*). None of the cytokines mentioned above affect the megakaryocyte maturational activity of rTPO.

30 The initial studies with rTPO clearly indicate that TPO predominantly affects the megakaryocytic lineage. However, like all other hematopoietic regulators, TPO affects other hematopoietic lineages as well. In the presence of EPO, rTPO has been shown to enhance erythroid burst (BFU-E) formation in human CD34+ colony assays ( M. Kobayashi *et al.*, *Blood* 86:2494 (1995); T. Papayannopoulou *et al.*, *Blood* 87:1833 (1996)). The burst promoting activity of rTPO is comparable to GM-CSF and KL and increases both the 35 number and size of BFU-E colonies (M. Kobayashi *et al.*, *supra*). In addition rTPO also stimulates CFU-E development, indicating that TPO acts on both early and late erythroid progenitors ( M. Kobayashi *et al.*, *supra*; T. Papayannopoulou *et al.*, *supra* ). In the absence of EPO, however, rTPO has no effect on erythropoiesis. An effect of rTPO on myeloid colony growth in normal hematopoietic cultures has not been demonstrated in vitro, however.

rTPO has a dramatic effect on platelet production when administered to normal animals. Pharmacological doses of recombinant forms of TPO cause as much as a 10 fold increase in platelet levels in mice and non-human primates (EF. Winton *et al.*, *Exp. Hematol.* 23:879 (1995); AM. Farese *et al.*, *Blood* 86:54 (1995); KH. Sprugel *et al.*, *Blood* 86(suppl 1):20 (1995); LA. Harker *et al.*, *Blood* 87:1833 (1996); K. 5 Kaushansky *et al.*, *Exp. Hematol.* 24:265 (1996); TR. Ulich *et al.*, *Blood* 87:5006 (1996); K. Ault *et al.*, *Blood* 86(suppl 1): 367 (1995); NC. Daw *et al.*, *Blood* 86 (suppl 1):5006 (1995)). This effect of rTPO is due to an increase in the synthesis of new platelets as reticulated platelets increase within 24 hours after rTPO administration (K. Ault *et al.*, *supra*). Preceding this effect is a dramatic increase in CFU-MK in both the marrow and spleen ( AM. Farese *et al.*, *supra*; K. Kaushansky *et al.*, *supra*; TR. Ulich *et al.*, *supra*). 10 Megakaryocytes from rTPO treated animals exhibit a higher mean ploidy and are larger in size than megakaryocytes from control animals. These later two observations again demonstrate the proliferative and maturational activities of TPO on the megakaryocytic lineage. Because the effect of TPO on megakaryocytes precedes its effect on platelet production it has been suggested that TPO primarily affects megakaryocyte progenitors rather than inducing platelet release from mature megakaryocytes (NC. Daw *et al.*, *supra*). No 15 significant effect on red blood cell (RBC) or white blood cell (WBC) production occurs in normal animals following rTPO administration. However, rTPO treatment caused an expansion of BFU-E and CFU-GM and a redistribution CFU-E in normal mice (K. Kaushansky *et al.*, *supra*) and expanded CFU-mixed in rhesus monkeys (AM. Farese *et al.*, *supra*).

Even though rTPO dramatically stimulates platelet production, it only has a modest effect on platelet 20 function. In vitro studies show that rTPO has no effect on platelet aggregation itself, but does enhance agonist induced aggregation (G. Montruccio *et al.*, *Blood* 87:2762 (1996); A. Oda *et al.*, *Blood* 87:4664 (1996); CF. Toombs *et al.*, *Thromb. Res.* 80:23 (1995); CF. Toombs *et al.*, *Blood* 86(suppl 1):369 (1995)). rTPO appears to sensitize platelets making them moderately more responsive to aggregation agonist. This raises the possibility that rTPO may have prothrombotic effects *in vivo*. However, an increase in thrombotic episodes in 25 animals treated with rTPO has never been observed, even when platelet levels were 4-10 fold above normal. In vivo thrombosis models also indicate that elevated platelet levels following rTPO treatment is not associated with an increase in platelet dependent thrombosis (LA. Harker *et al.*, *supra*; CF. Toombs *et al.*, *supra*). These results indicate that stimulation of platelet production by rTPO will unlikely be associated with an increase in thrombo-occlusive events.

30 The involvement of c-Mpl and TPO in the control of platelet production and its effect on other hematopoietic lineages is further demonstrated by the phenotype of mice deficient in either the c-mpl or the TPO genes (WS. Alexander *et al.*, *Blood* 87:2162 (1996); FJ. de Sauvage *et al.*, *J.Exp.Med.* 183:651 (1996); AL. Gurney *et al.*, *Science* 265:1445 (1994)). In both cases a dramatic 85 to 90% drop in platelet counts is observed with a similar decrease of megakaryocytes in the spleen and bone marrow. In addition, the 35 megakaryocytes of the knockout mice are smaller and exhibit a lower ploidy than those of control mice. The similarity in phenotype observed for these knock-outs (KO) indicates that the system is non-redundant and that there is probably only one receptor for TPO and one ligand for c-Mpl. Although the platelet number is reduced in the KO mice their platelets appear normal, both structurally and functionally, and are sufficient to prevent overt bleeding. The genes and factors involved in the production of this basal level of platelets and

megakaryocytes still remain to be identified. However, treatment of either the TPO or c-mpl knockout mice with other cytokines with megakaryopoietic activity (IL-6, IL-11 and stem cell factor) results in a modest stimulation of platelet production (AL. Gurney *et al.*, *supra*). This suggest that these cytokines do not require TPO or c-mpl to exert their thrombopoietic activity and, therefore, may be involved in the maintenance of a basal level of megakaryocytes and platelets.

Comparison of CFU-megakaryocyte (CFU-Meg) from TPO or c-mpl deficient and normal mice shows that the number of megakaryocytes progenitors is decreased in both knock-outs compared to control, suggesting that TPO acts on very early megakaryocyte progenitors. In addition, both erythroid and myeloid progenitors are also reduced in the TPO and c-Mpl knockout mice (WS. Alexander *et al.*, *supra*; K. Carver-Moore *et al.*, 88:803 (1996)). This reduction in progenitors from all lineages indicates that TPO probably acts on a very early pluripotent progenitor cell. The involvement of TPO and c-Mpl at an early stage of hematopoiesis correlates with the detection of c-Mpl expression in AA4+ Sca+ murine stem cell population (FC. Zeigler *et al.*, *supra*). The effect of TPO on this most primitive stem cell population still remains to be investigated, however, preliminary data indicate that TPO may directly affect the proliferation of primitive murine hematopoietic stem or progenitor cells (E. Stinicka *et al.*, *Blood* 87:4998 (1996); M. Kobayashi *et al.*, *Blood* 88:429 (1996); H. Ku *et al.*, *Blood* 87:4544 (1996)). This, in part, may explain the effect TPO has on erythropoiesis and myelopoiesis *in vitro* and *in vivo*.

It has long been observed that an inverse correlation exists between plasma megakaryopoietic and thrombopoietic activity and platelet levels (reviewed in TP. McDonald, *Am. J. Pediatr. Hematol./Oncol.* 14:8 (1992)). TPO specific ELISAs and cell proliferation assays have now confirmed that TPO levels increase and decrease inversely with platelet mass (JL. Nichol *et al.*, *supra*; EVB. Emmons *et al.*, *Blood* 87:4068 (1996); H. Oh *et al.*, *Blood* 87:4918 (1996); M. Chang *et al.*, *Blood* 86(suppl 1):368 (1995)). Unlike erythropoietin, however, TPO does not appear to be regulated at the transcriptional level, but rather by platelet mass. This was initially proposed de Gabriele and Pennington (G. de Gabriele *et al.*, *Br. J. Haematol.* 13:202 (1967); G. de Gabriele *et al.*, *Br. J. Haematol.* 13:210 (1967)) and subsequently confirmed by Kuter and Rosenberg (DJ. Kuter *et al.*, *Blood* 84:1464 (1994)) who showed direct regulation of circulating TPO levels by exogenously administering platelets to thrombocytopenic mice. More recently, it was demonstrated that TPO mRNA levels in thrombocytopenic mice are not increased even though TPO levels are elevated by at least 10 fold (PJ. Fielder *et al.*, *Blood* 87:2154 (1996); R. Stoffel *et al.*, *Blood* 87:567 (1996)). In addition, the gene dosage effect observed in TPO heterozygous knockout mice refute the regulation of TPO production by platelet mass (FJ. de Sauvage *et al.*, *supra*). Taken together, these results strongly support the hypothesis that TPO expression is constitutive and it is the sequestering by platelets that regulates TPO levels. Platelets bind TPO with high affinity (Kd(100-400pM) and internalize and degrade TPO (PJ. Fielder *et al.*, *supra*). Platelets from c-Mpl knockout mice do not bind TPO and the clearance of TPO by these mice is 5 fold slower than that observed for wild type mice (PJ. Fielder *et al.*, *supra*). These results indicate that TPO clearance is mediated by platelet binding via c-Mpl. It is also likely that megakaryocyte mass plays a role in regulating circulating TPO levels. This is supported by the observation that both ITP patients and mice deficient in the NF-E2 transcription factor are highly thrombocytopenic, exhibit megakaryocytosis, but have normal TPO levels (EVB. Emmons *et al.*, *supra*; RA. Shivdasani *et al.*, *Cell* 81:695 (1995)). In situ studies with radiolabeled TPO show that marrow

megakaryocytes of the NF-E2 mice bind significant amounts of labeled TPO (RA. Shivdasani *et al.*, *Blood* submitted (1996)). The phenotype of the ITP and NF-E2 knockout mice, therefore, suggest that binding of TPO to megakaryocytes may also regulate TPO levels.

The dramatic effect of rTPO on platelet production in normal mice and monkeys and subsequent clinical trials indicate that rTPO is clinically useful in alleviating thrombocytopenia associated with myelosuppressive and myeloablative therapies for cancer patients. In several myelosuppressive and myeloablative murine and monkey preclinical models recombinant forms of TPO have been shown to significantly affect platelet recovery. In mice treated with carboplatin and sublethal irradiation in combination (JP Leonard *et al.*, *Blood* 83:1499 (1994)), daily treatment with rTPO both reduced the severity of the platelet nadir and accelerated platelet recovery by 10-12 days when compared to excipient treated animals (GR. Thomas *et al.*, *supra*; K. Kaushansky *et al.*, *supra*; MM. Hokom *et al.*, *Blood* 86:4486 (1995)). Similar results were obtained in a murine sublethal irradiation model (GR. Thomas *et al.*, *supra*). In murine myeloablative transplantation models rTPO has been shown to reduce the extent of the nadir and accelerate platelet recovery by 2-3 weeks (GR. Thomas *et al.*, *supra*; K. Kabaya *et al.*, *Blood* 86(suppl 1):114 (1995); G. Molineux *et al.*, *Blood* 86(suppl 1):227 (1995)). Treatment of sublethally irradiated rhesus monkeys with rTPO accelerated platelet recovery by 3 weeks and prevented platelet nadirs below 40,000 (AM. Farese *et al.*, *J. Clin. Invest.* 97:2145 (1996); KJ. Neelis *et al.*, *Blood* 86(suppl 1):256 (1995)). Even more impressively, rTPO completely prevented post-chemotherapy thrombocytopenia following the treatment of rhesus monkeys with hepsulfam (AM. Farese *et al.*, *supra*). In contrast to these promising results, two groups have reported that rTPO had no effect on the hematopoietic recovery of lethally irradiated mice or monkeys rescued with a marrow transplant (KJ. Neelis *et al.*, *supra*; WE. Fibbe *et al.*, *Blood* 86:3308 (1995)). The reason for this discrepancy is unclear, however it is possible that lethal radiation may destroy stromal cells or components essential for TPO activity *in vivo*. In support of this, lethally irradiated mice transplanted with marrow cells from rTPO treated donor mice show accelerated recovery of platelets and RBCs, however, post-transplant administration of rTPO had no further effect on this accelerated recovery (WE. Fibbe *et al.*, *supra*). This result suggests that although the transplanted cell population was enriched for megakaryocyte progenitors, TPO had no effect on these progenitors in a lethally irradiated marrow.

Although rTPO only modestly affects erythroid and myeloid lineages in normal mice it dramatically accelerates the recovery of all progenitor classes in myelosuppressed mice and monkeys resulting in a significant acceleration of RBC and WBC recovery (K. Kaushansky *et al.*, *supra*; AM. Farese *et al.*, *supra*; K. Kaushansky *et al.*, *J. Clin. Invest.* 96:1683 (1995)). The effect of rTPO on neutrophil recovery has been shown to be additive to that of G-CSF (AM. Farese *et al.*, *supra*). These results indicate that the clinical utility of rTPO may be broader than originally anticipated.

The difference between the effect of rTPO on hematopoiesis in normal and myelosuppressed animals is likely due to the change in the cytokine environment that occurs following myelosuppressive therapy. It is likely that elevated levels of EPO, G-CSF or other cytokines essential for erythropoiesis and myelopoiesis present following myelosuppressive treatment interact with rTPO to have a multilineage effect (K. Kaushansky *et al.*, *supra*). In normal mice the level of these cytokines are insufficient and the effects of rTPO on erythroid and myeloid lineages are less significant. This hypothesis is supported by the above mentioned synergistic

interaction of rTPO and EPO to stimulate in vitro erythropoiesis (ES. Choi *et al.*, *supra*). It has also been proposed that production of hemopoietic factors from megakaryocytes themselves may also play a role in the multilineage effect of rTPO (AM. Farese *et al.*, *supra*).

5 In the above mentioned animal studies rTPO was administered daily for 14-28 days, which was based on previous experience in dosing other hematopoietic growth factors. However, it has recently been shown that a single dose of rTPO following myelosuppressive treatment of mice with carboplatin and sublethal irradiation is as effective as multiple doses in reducing nadirs and accelerating platelet and RBC recovery (GR. Thomas *et al.*, *supra*). This effect is likely due to the potency and long half life of rTPO

10 (GR. Thomas *et al.*, *supra*). This is supported by the fact that single doses of unglycosylated rTPO153 are not effective in this model. These observations indicate that the frequency of rTPO dosing required to affect hematopoietic recovery following myelosuppressive treatment may be significantly less than that for other currently used cytokines

15 Early results from human clinical trials show that rTPO also stimulates platelet production in humans. In phase I trials, a pegylated and truncated form of rTPO (MGDF) administered daily for 10 days at 0.03 - 5.0 µg/kg to cancer patients prior to chemotherapy caused up to a four fold increase in circulating platelet levels (R. Basser *et al.*, *Blood* 86(suppl 1): 257 (1995); JEJ. Rasko *et al.*, *Blood* 86(suppl 1):497 (1995)). Similarly, patients given a single dose of rTPO had platelet levels increase by four fold (S. Vaden-Raj *et al.*, Stimulation of megakaryocyte and platelet production by a single dose of recombinant human thrombopoietin in cancer patients. Submitted. (1996)). In both studies platelet increases are observed by day four and peak about 12-16 days later. No drug related toxicity's were reported and, although platelet levels greater than  $1 \times 10^6/\mu\text{l}$  were observed in some of the patients, no thrombotic events were observed. This indicates that TPO will be well tolerated in humans. In myelosuppressed patients, pegylated rTPO153(MGDF) given post chemotherapy has been shown to reduce the extent of the platelet nadir following chemotherapy (G. Begley *et al.*, *Proceedings of ASCO* 15:271 (1996); M. Fanucchi *et al.*, *Proceedings of ASCO* 15:271 (1996)). As seen in the preclinical 20 animals studies, TPO also expanded marrow progenitors of megakaryocyte, erythroid, myeloid and multipotential lineages (S. Vaden-Raj *et al.*, *supra*). This later observation suggests that rTPO may be useful as a priming agent.

25 It is believed that the proliferation and maturation of hematopoietic cells is tightly regulated by factors that positively or negatively modulate pluripotential stem cell proliferation and multilineage differentiation. 30 These effects are mediated through the high-affinity binding of extracellular protein factors (ligands) to specific cell surface receptors. These cell surface receptors share considerable homology and are generally classified as members of the cytokine receptor superfamily. Members of the superfamily include receptors for: IL-2 (b and g chains) (Hatakeyama *et al.*, *Science*, 244:551-556 (1989); Takeshita *et al.*, *Science*, 257:379-382 (1991)), IL-3 (Itoh *et al.*, *Science*, 247:324-328 (1990); Gorman *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:5459-5463 (1990); 35 Kitamura *et al.*, *Cell*, 66:1165-1174 (1991a); Kitamura *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:5082-5086 (1991b)), IL-4 (Mosley *et al.*, *Cell*, 59:335-348 (1989)), IL-5 (Takaki *et al.*, *EMBO J.*, 9:4367-4374 (1990); Tavernier *et al.*, *Cell*, 66:1175-1184 (1991)), IL-6 (Yamasaki *et al.*, *Science*, 241:825-828 (1988); Hibi *et al.*, *Cell*, 63:1149-1157 (1990)), IL-7 (Goodwin *et al.*, *Cell*, 60:941-951 (1990)), IL-9 (Renault *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:5690-5694 (1992)), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Gearing

*et al.*, *EMBO J.*, 8:3667-3676 (1991); Hayashida *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:8702-8706 (1990)), granulocyte colony-stimulating factor (G-CSF) (Fukunaga *et al.*, *Cell*, 61:341-350 (1990a); Fukunaga *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:8702-8706 (1990b); Larsen *et al.*, *J. Exp. Med.*, 172:1559-1570 (1990)), EPO (D'Andrea *et al.*, *Cell*, 57:277-285 (1989); Jones *et al.*, *Blood*, 76:31-35 (1990)), Leukemia inhibitory factor (LIF) (Gearing *et al.*, *EMBO J.*, 10:2839-2848 (1991)), oncostatin M (OSM) (Rose *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:8641-8645 (1991)) and also receptors for prolactin (Boutin *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:7744-7748 (1988); Edery *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:2112-2116 (1989)), growth hormone (GH) (Leung *et al.*, *Nature*, 330:537-543 (1987)) and ciliary neurotrophic factor (CNTF) (Davis *et al.*, *Science*, 253:59-63 (1991)).

10 Members of the cytokine receptor superfamily may be grouped into three functional categories (for review see Nicola *et al.*, *Cell*, 67:1-4 (1991)). The first class comprises single chain receptors, such as erythropoietin receptor (EPO-R) or granulocyte colony stimulating factor receptor (G-CSF-R), which bind ligand with high affinity via the extracellular domain and also generate an intracellular signal. A second class of receptors, so called  $\alpha$ -subunits, includes interleukin-6 receptor (IL6-R), granulocyte-macrophage colony stimulating factor receptor (GM-CSF-R), interleukin-3 receptor (IL3-Ra) and other members of the cytokine receptor superfamily. These  $\alpha$ -subunits bind ligand with low affinity but cannot transduce an intracellular signal. A high affinity receptor capable of signaling is generated by a heterodimer between an  $\alpha$ -subunit and a member of a third class of cytokine receptors, termed  $\beta$ -subunits, *e.g.*,  $\beta_C$ , the common  $\beta$ -subunit for the three  $\alpha$ -subunits of IL-3-R, IL-5-R and GM-CSF-R (Nicola N. A. *et. al.* *Cell* 67:1-4 (1991))

15 20 Evidence that *mpl* is a member of the cytokine receptor superfamily comes from sequence homology (Gearing, *EMBO J.*, 8:3667-3676 (1988); Bazan, *Proc. Natl. Acad. Sci. USA*, 87:6834-6938 (1990); Davis *et al.*, *Science*, 253:59-63 (1991) and Vigon *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:5640-5644 (1992)) and its ability to transduce proliferative signals.

25 30 Deduced protein sequence from molecular cloning of murine *c-mpl* reveals this protein is homologous to other cytokine receptors. The extracellular domain contains 465 amino acid residues and is composed of two subdomains each with four highly conserved cysteines and a particular motif in the N-terminal subdomain and in the C-terminal subdomain. The ligand-binding extracellular domains are predicted to have similar double  $\beta$ -barrel fold structural geometries. This duplicated extracellular domain is highly homologous to the signal transducing chain common to IL-3, IL-5 and GM-CSF receptors as well as the low-affinity binding domain of LIF (Vigon *et al.*, *Oncogene*, 8:2607-2615 (1993)). Thus *mpl* may belong to the low affinity ligand binding class of cytokine receptors.

35 A comparison of murine *mpl* and mature human *mpl* P, reveals these two proteins show 81% sequence identity. More specifically, the N-terminus and C-terminus extracellular subdomains share 75% and 80% sequence identity respectively. The most conserved *mpl* region is the cytoplasmic domain showing 91% amino acid identity, with a sequence of 37 residues near the transmembrane domain being identical in both species. Accordingly, *mpl* is reported to be one of the most conserved members of the cytokine receptor superfamily (Vigon *supra*).

Activation of certain hematopoietic receptors is believed to cause one or more effects including; stimulation of proliferation, stimulation of differentiation, stimulation of growth and inhibition of apoptosis

(Libol *et al* Proc. Natl. Acad. Sci. 248:378 (1993). Activation of hematopoietic receptors upon ligand binding may be due to dimerization of two or more copies of the receptor. In addition to the naturally occurring ligand causing this dimerization, agonist antibodies may also activate receptors by crosslinking or otherwise causing dimerization of a receptor. Such antibodies are useful for the same indications as the natural ligand and may

5 have advantageous properties such as a longer half-life. An example of a monoclonal antibody to a cytokine receptor that activates the erythropoietin receptor (EPO-R) is described in WO 96/03438 (published 8 February 1996). These agonist antibodies to EPO-R are about 3-4 orders of magnitude weaker in activity based on weight than the natural EPO ligand.

There is a current and continuing need to isolate and identify molecules, especially antibodies, 10 fragments and derivatives thereof, capable of stimulating proliferation, differentiation and maturation and/or modulation of apoptosis of cells, for example hematopoietic cells, including megakaryocytes or their predecessors for therapeutic use in the treatment of hematopoietic disorders including thrombocytopenia.

#### Summary Of The Invention

Accordingly, It is an object of this invention to obtain a pharmaceutically or essentially pure antibody 15 or fragments or derivatives thereof capable of stimulating proliferation, differentiation and/or maturation of hematopoietic cells, including megakaryocytes or their predecessors, or to modulate apoptosis of hematopoietic cells.

It is a specific object of the present invention to isolate antibody ligands capable of binding *in vivo* a hematopoietic growth factor superfamily receptor and to activate the receptor, the antibody having a biological 20 activity equal to or not less than 2 orders of magnitude below that of the naturally occurring ligand on a weight basis .

It is also an object of the present invention to isolate antibody ligands capable of binding to and activating any of the three functional categories of cytokine superfamily receptors (see Nicola *et al.*, *Cell*, 67:1-4 (1991)).

25 In one embodiment, the objects of the invention are achieved by providing an antibody or fragment thereof that activates a hematopoietic growth factor superfamily receptor having a biological activity within 2 orders of magnitude (100), preferably within one order of magnitude (10), of the natural ligand on a weight basis. Preferably, the antibody activates the thrombopoietin (TPO) receptor. This antibody, referred to as an agonist antibody, activates a thrombopoietin receptor which preferably comprises a mammalian c-mpl, more 30 preferably human c-mpl. Usually the antibody will be a full length antibody such as an IgG antibody. Suitable representative fragment agonist antibodies include Fv, ScFv, Fab, F(ab')<sub>2</sub> fragments, as well as diabodies and linear antibodies. These fragments may be fused to other sequences including, for example, the F" or Fc region 35 of an antibody, a "leucine zipper" or other sequences including pegylated sequences or Fc mutants used to improve or modulate half-life. Normally the antibody is a human antibody and may be a non-naturally occurring antibody, including affinity matured antibodies. Representative antibodies that activate c-mpl are selected from the group 12E10, 12B5, 10F6 and 12D5, and affinity matured derivatives thereof. Other preferred agonist antibodies to c-mpl are selected from the group consisting of Ab1, Ab2, Ab3, Ab4, Ab5 and Ab6, wherein each Ab1-Ab6 contains a VH and VL chain and each VH and VL chain contains complementarity determining region (CDR) amino acid sequences designated CDR1, CDR2 and CDR3

separated by framework amino acid sequences, the amino acid sequence of each CDR in each VH and VL chain of Ab1-Ab6 is shown in Table 1.

Table 1

Ab1:	VH CDR1	VH CDR2	VH CDR3
	DNA (SEQ ID NO: 1) protein (SEQ ID NO: 2)	(SEQ ID NO: 3) (SEQ ID NO: 4)	(SEQ ID NO: 5) (SEQ ID NO: 6)
	VL CDR1	VL CDR2	VL CDR3
	DNA (SEQ ID NO: 7) protein (SEQ ID NO: 8)	(SEQ ID NO: 9) (SEQ ID NO: 10)	(SEQ ID NO: 11) (SEQ ID NO: 12)
Ab2:	VH CDR1	VH CDR2	VH CDR3
	DNA (SEQ ID NO: 13) protein (SEQ ID NO: 14)	(SEQ ID NO: 15) (SEQ ID NO: 16)	(SEQ ID NO: 17) (SEQ ID NO: 18)
	VL CDR1	VL CDR2	VL CDR3
	DNA (SEQ ID NO: 19) protein (SEQ ID NO: 20)	(SEQ ID NO: 21) (SEQ ID NO: 22)	(SEQ ID NO: 23) (SEQ ID NO: 24)
Ab3:	VH CDR1	VH CDR2	VH CDR3
	DNA (SEQ ID NO: 25) protein (SEQ ID NO: 26)	(SEQ ID NO: 27) (SEQ ID NO: 28)	(SEQ ID NO: 29) (SEQ ID NO: 30)
	VL CDR1	VL CDR2	VL CDR3
	DNA (SEQ ID NO: 19) protein (SEQ ID NO: 20)	(SEQ ID NO: 21) (SEQ ID NO: 22)	(SEQ ID NO: 23) (SEQ ID NO: 24)
Ab4:	VH CDR1	VH CDR2	VH CDR3
	DNA (SEQ ID NO: 25) protein (SEQ ID NO: 26)	(SEQ ID NO: 31) (SEQ ID NO: 32)	(SEQ ID NO: 33) (SEQ ID NO: 34)
	VL CDR1	VL CDR2	VL CDR3
	DNA (SEQ ID NO: 35) protein (SEQ ID NO: 20)	(SEQ ID NO: 21) (SEQ ID NO: 22)	(SEQ ID NO: 23) (SEQ ID NO: 24)
Ab5:	VH CDR1	VH CDR2	VH CDR3
	DNA (SEQ ID NO: 36) protein (SEQ ID NO: 37)	(SEQ ID NO: 38) (SEQ ID NO: 39)	(SEQ ID NO: 40) (SEQ ID NO: 41)

	VL CDR1	VL CDR2	VL CDR3
DNA	(SEQ ID NO: 19)	(SEQ ID NO: 21)	(SEQ ID No: 23)
protein	(SEQ ID NO: 20)	(SEQ ID NO: 22)	(SEQ ID No: 24)
Ab6:	VH CDR1	VH CDR2	VH CDR3
DNA	(SEQ ID NO: 42)	(SEQ ID NO: 44)	(SEQ ID No: 46)
protein	(SEQ ID NO: 43)	(SEQ ID NO: 45)	(SEQ ID No: 47)
	VL CDR1	VL CDR2	VL CDR3
DNA	(SEQ ID NO: 48)	(SEQ ID NO: 50)	(SEQ ID No: 52)
protein	(SEQ ID NO: 49)	(SEQ ID NO: 51)	(SEQ ID No: 53)

Other preferred c-mpl agonist antibodies of this invention include those that activate platelets in a manner similar to TPO or in a manner similar to ADP, collagen and the like. Optionally the c-mpl agonist antibodies of this invention do not activate platelets. The c-mpl agonist antibodies of this invention are used in a manner similar to TPO.

5 In another embodiment, substantially pure single chain antibodies are provided which bind to and act as agonist or antagonist antibodies to a cytokine receptor or to a kinase receptor.

The invention also provides a method of obtaining these antibodies, in particular a method of screening a library of phage displayed antibodies, preferably human single chain antibodies.

Brief Description Of The Drawings

10 Fig. 1 shows examples of single chain antibody (scFv) fragments denominated 10F6, 5E5, 10D10, 12B5, 12D5 and 12E10 having sequences for CDRs and framework regions.

Fig. 2 illustrates a method for the construction of a phage library containing single-chain antibodies fused to a coat protein of a phage.

15 Fig. 3 shows a single-chain antibody displayed as a fusion protein on coat protein 3 of a filamentous phage.

Fig. 4 illustrates a method of selecting scFv in a phage library by one or more binding selection cycles.

Fig. 5 illustrates a method of panning high affinity phage using biotinylated antigen and streptavidin coated paramagnetic beads.

Fig. 6 shows a process for identifying c-mpl binding phage using a phage ELISA method.

20 Fig. 7 illustrates DNA fingerprinting of clones to determine diversity by BstNI restriction enzyme analysis.

Fig. 8A-C show a typical BstNI analysis on a 3% agarose gel; see Example 2.

Fig. 9 shows the results of agonist antibodies relative to TPO in the KIRA-ELISA assay.

Fig. 10A-F show the results of TPO-antibody competitive binding assays for HU-03 cells. See 25 Example 1.

Fig. 11 shows activity for MuSK agonist antibodies of Example 5.

Detailed Description Of The Preferred Embodiments

I. Definitions

In general, the following words or phrases have the indicated definition when used in the description, 5 examples, and claims.

The terms "agonist" and "agonistic" when used herein refer to or describe a molecule which is capable of, directly or indirectly, substantially inducing, promoting or enhancing cytokine biological activity or cytokine receptor activation.

"Agonist antibodies"(aAb) are antibodies or fragments thereof that possess the property of binding to 10 a cytokine superfamily receptor and causing the receptor to transduce a survival, proliferation, maturation and/or differentiation signal. Included within the definition of transducing a survival signal is a signal which modulates cell survival or death by apoptosis. To be therapeutically useful the agonist antibodies of this invention will be capable of inducing or causing survival, proliferation, maturation or differentiation at a concentration equal to or not less than 2 orders of magnitude (100-fold) below that of the natural *in vivo* ligand 15 on a weight basis.

"Activate a receptor", as used herein, is used interchangeably with transduce a growth, survival, proliferation, maturation and/or differentiation signal.

"Activate platelets", as used herein, means to stimulate platelets to make them more likely to aggregate 20 by comparison to unactivated platelets. For example, ADP and collagen are substances known to activate platelets.

"Affinity matured antibodies" are antibodies that have had their binding affinity and/or biological activity increased by altering the type or location of one or more residues in the variable region. An example of alteration is a mutation which may be in either a CDR or a framework region. An affinity matured antibody will typically have its binding affinity increased above that of the isolated or natural antibody or fragment 25 thereof by from 2 to 500 fold. Preferred affinity matured antibodies will have nanomolar or even picomolar affinities to the receptor antigen. Affinity matured antibodies are produced by procedures known in the art. Marks, J. D. *et al.* *Bio/Technology* 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by; Barbas, C. F. *et al.* *Proc Nat. Acad. Sci, USA* 91:3809-3813 (1994), Schier, R. *et al.* *Gene* 169:147-155 (1995), Yelton, D. E. *et al.* *J. 30 Immunol.* 155:1994-2004 (1995), Jackson, J.R. *et al.* *J. Immunol.* 154(7):3310-9 (1995), and Hawkins, R.E. *et al.* *J. Mol. Biol.* 226:889-896 (1992).

"Cytokine" is a generic term for proteins released by one cell population which act on another cell as 35 intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone, insulin-like growth factors, human growth hormone, N-methionyl human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin, proinsulin, relaxin, prorelaxin, glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and leutinizing hormone (LH), hematopoietic growth factor, hepatic growth factor, fibroblast growth factor, prolactin, placental lactogen, tumor necrosis factor-a (TNF-a and TNF-b) mullerian-inhibiting substance, mouse gonadotropin-associated peptide, inhibin, activin, vascular endothelial

growth factor, integrin, nerve growth factors such as NGF- $\beta$ , platelet-growth factor, transforming growth factors (TGFs) such as TGF- $\alpha$  and TGF- $\beta$ , insulin-like growth factor-I and -II, erythropoietin (EPO), osteoinductive factors, interferons such as interferon- $\alpha$ , - $\beta$ , and - $\gamma$ , colony stimulating factors (CSFs) such as

5 macrophage-CSF (M-CSF), granulocyte-macrophage-CSF (GM-CSF), and granulocyte-CSF (G-CSF), thrombopoietin (TPO), interleukins (IL's) such as IL-1, IL-1a, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12 and other polypeptide factors including LIF, SCF, and kit-ligand. As used herein the foregoing terms are meant to include proteins from natural sources or from recombinant cell culture. Similarly, the terms are intended to include biologically active equivalents; e.g., differing in amino acid sequence by one or more amino acids or in type or extent of glycosylation.

10 "Cytokine superfamily receptors" and "hematopoietic growth factor superfamily receptors" are used interchangeably herein and are a group of closely related glycoprotein cell surface receptors that share considerable homology including frequently a WSXWS domain and are generally classified as members of the cytokine receptor superfamily (see e.g. Nicola *et al.*, *Cell*, 67:1-4 (1991) and Skoda, R.C. *et al.* *EMBO J.* 12:2645-2653 (1993)). Generally, these receptors are interleukins (IL) or colony-stimulating factors (CSF).

15 Members of the superfamily include, but are not limited to, receptors for: IL-2 ( $\beta$  and  $\gamma$  chains) (Hatakeyama *et al.*, *Science*, 244:551-556 (1989); Takeshita *et al.*, *Science*, 257:379-382 (1991)), IL-3 (Itoh *et al.*, *Science*, 247:324-328 (1990); Gorman *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:5459-5463 (1990); Kitamura *et al.*, *Cell*, 66:1165-1174 (1991a); Kitamura *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:5082-5086 (1991b)), IL-4 (Mosley *et al.*, *Cell*, 59:335-348 (1989), IL-5 (Takaki *et al.*, *EMBO J.*, 9:4367-4374 (1990); Tavernier *et al.*, *Cell*, 66:1175-20 1184 (1991)), IL-6 (Yamasaki *et al.*, *Science*, 241:825-828 (1988); Hibi *et al.*, *Cell*, 63:1149-1157 (1990)), IL-7 (Goodwin *et al.*, *Cell*, 60:941-951 (1990)), IL-9 (Renault *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:5690-5694 (1992)), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Gearing *et al.*, *EMBO J.*, 8:3667-3676 (1991); Hayashida *et al.*, *Proc. Natl. Acad. Sci. USA*, 244:9655-9659 (1990)), granulocyte colony-stimulating factor (G-CSF) (Fukunaga *et al.*, *Cell*, 61:341-350 (1990a); Fukunaga *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:8702-8706 (1990b); Larsen *et al.*, *J. Exp. Med.*, 172:1559-1570 (1990)), EPO (D'Andrea *et al.*, *Cell*, 57:277-285 (1989); Jones *et al.*, *Blood*, 76:31-35 (1990)), Leukemia inhibitory factor (LIF) (Gearing *et al.*, *EMBO J.*, 10:2839-2848 (1991)), oncostatin M (OSM) (Rose *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:8641-8645 (1991)) and also receptors for prolactin (Boutin *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:7744-7748 (1988); Edery *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:2112-2116 (1989)), growth hormone (GH) (Leung *et al.*, *Nature*, 330:537-543 (1987)), ciliary neurotrophic factor (CNTF) (Davis *et al.*, *Science*, 253:59-63 (1991) and c-Mpl (M. Souyri *et al.*, *Cell* 63:1137 (1990); I. Vigon *et al.*, *Proc. Natl. Acad. Sci.* 89:5640 (1992)).

"Thrombocytopenia" in humans is defined as a platelet count below  $150 \times 10^9$  per liter of blood.

30 "Thrombopoietic activity" is defined as biological activity that consists of accelerating the proliferation, differentiation and/or maturation of megakaryocytes or megakaryocyte precursors into the platelet producing form of these cells. This activity may be measured in various assays including an *in vivo* mouse platelet rebound synthesis assay, induction of platelet cell surface antigen assay as measured by an anti-platelet immunoassay (anti-GPIIbIIIa) for a human leukemia megakaryoblastic cell line (CMK), and induction of polyploidization in a megakaryoblastic cell line (DAMI). A "thrombopoietin receptor" is a mammalian

polypeptide receptor which, when activated by a ligand binding thereto, includes, causes or otherwise gives rise to "thrombopoietic activity" in a cell or mammal, including a human.

"Control sequences" when referring to expression means DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for 5 prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

"Operably linked" when referring to nucleic acids means that the nucleic acids are placed in a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory 10 leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, 15 enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adapters or linkers are used in accord with conventional practice.

"Exogenous" when referring to an element means a nucleic acid sequence that is foreign to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is ordinarily 20 not found.

"Cell," "cell line," and "cell culture" are used interchangeably herein and such designations include all progeny of a cell or cell line. Thus, for example, terms like "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent 25 mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

"Plasmids" are autonomously replicating circular DNA molecules possessing independent origins of replication and are designated herein by a lower case "p" preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted 30 basis, or can be constructed from such available plasmids in accordance with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

"Restriction enzyme digestion" when referring to DNA means catalytic cleavage of internal phosphodiester bonds of DNA with an enzyme that acts only at certain locations or sites in the DNA sequence. Such enzymes are called "restriction endonucleases". Each restriction endonuclease recognizes a specific DNA 35 sequence called a "restriction site" that exhibits two-fold symmetry. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements as established by the enzyme suppliers are used. Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters representing the microorganism from which each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1  $\mu$ g of plasmid

or DNA fragment is used with about 1-2 units of enzyme in about 20  $\mu$ l of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation of about 1 hour at 37°C is ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein or polypeptide is removed by extraction with phenol and chloroform, and the digested nucleic acid is 5 recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme may be followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction-cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional as 10 described in sections 1.56-1.61 of Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989).

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the 15 gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see Lawn *et al.*, *Nucleic Acids Res.*, 9:6103-6114 (1981), and Goeddel *et al.*, *Nucleic Acids Res.*, 8:4057 (1980).

"Southern analysis" or "Southern blotting" is a method by which the presence of DNA sequences in a restriction endonuclease digest of DNA or DNA-containing composition is confirmed by hybridization to a known, labeled oligonucleotide or DNA fragment. Southern analysis typically involves electrophoretic separation of DNA digests on agarose gels, denaturation of the DNA after electrophoretic separation, and transfer of the DNA to nitrocellulose, nylon, or another suitable membrane support for analysis with a radiolabeled, biotinylated, or enzyme-labeled probe as described in sections 9.37-9.52 of Sambrook *et al.*, *supra*.

25 "Northern analysis" or "Northern blotting" is a method used to identify RNA sequences that hybridize to a known probe such as an oligonucleotide, DNA fragment, cDNA or fragment thereof, or RNA fragment. The probe is labeled with a radioisotope such as  $^{32}$ P, or by biotinylation, or with an enzyme. The RNA to be analyzed is usually electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe, using standard techniques 30 well known in the art such as those described in sections 7.39-7.52 of Sambrook *et al.*, *supra*.

"Ligation" is the process of forming phosphodiester bonds between two nucleic acid fragments. For ligation of the two fragments, the ends of the fragments must be compatible with each other. In some cases, the ends will be directly compatible after endonuclease digestion. However, it may be necessary first to convert the staggered ends commonly produced after endonuclease digestion to blunt ends to make them compatible for 35 ligation. For blunting the ends, the DNA is treated in a suitable buffer for at least 15 minutes at 15°C with about 10 units of the Klenow fragment of DNA polymerase I or T4 DNA polymerase in the presence of the four deoxyribonucleotide triphosphates. The DNA is then purified by phenol-chloroform extraction and ethanol precipitation. The DNA fragments that are to be ligated together are put in solution in about equimolar amounts. The solution will also contain ATP, ligase buffer, and a ligase such as T4 DNA ligase at about 10

units per 0.5  $\mu$ g of DNA. If the DNA is to be ligated into a vector, the vector is first linearized by digestion with the appropriate restriction endonuclease(s). The linearized fragment is then treated with bacterial alkaline phosphatase or calf intestinal phosphatase to prevent self-ligation during the ligation step.

“Preparation” of DNA from cells means isolating the plasmid DNA from a culture of the host cells.

5 Commonly used methods for DNA preparation are the large- and small-scale plasmid preparations described in sections 1.25-1.33 of Sambrook *et al.*, *supra*. After preparation of the DNA, it can be purified by methods well known in the art such as that described in section 1.40 of Sambrook *et al.*, *supra*.

“Oligonucleotides” are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, 10 using solid-phase techniques such as described in EP 266,032 published 4 May 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler *et al.*, *Nucl. Acids Res.*, 14:5399-5407 (1986)). Further methods include the polymerase chain reaction defined below and other autoprimer methods and oligonucleotide syntheses on solid supports. All of these methods are described in Engels *et al.*, *Agnew. Chem. Int. Ed. Engl.*, 28:716-734 (1989). These methods are used if the entire nucleic acid sequence of the gene is 15 known, or the sequence of the nucleic acid complementary to the coding strand is available. Alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue. The oligonucleotides are then purified on polyacrylamide gels.

“Polymerase chain reaction” or “PCR” refers to a procedure or technique in which minute amounts of 20 a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195 issued 28 July 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, 25 specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, *etc.* See generally Mullis *et al.*, *Cold Spring Harbor Symp. Quant. Biol.*, 51:263 (1987); Erlich, ed., *PCR Technology*, (Stockton Press, NY, 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample comprising the use of a known nucleic acid as a primer and a nucleic acid polymerase to amplify or 30 generate a specific piece of nucleic acid.

“Native antibodies and immunoglobulins” are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced 35 intrachain disulfide bridges. Each heavy chain has at one end a variable domain ( $V_H$ ) followed by a number of constant domains. Each light chain has a variable domain at one end ( $V_L$ ) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid

residues are believed to form an interface between the light and heavy chain variable domains (Clothia *et al.*, *J. Mol. Biol.*, 186:651-663 (1985); Novotny and Haber, *Proc. Natl. Acad. Sci. USA*, 82:4592-4596 (1985)).

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed through the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a  $\beta$ -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the  $\beta$ -sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, National Institute of Health, Bethesda, MD (1987)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

Papain digestion of antibodies produces two identical antigen binding fragments, called "Fab" fragments, each with a single antigen binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an  $F(ab')_2$  fragment that has two antigen combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the  $V_H$ - $V_L$  dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group.  $F(ab')_2$  antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other, chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda ( $\lambda$ ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. The heavy chain constant domains that correspond to the different classes of

immunoglobulins are called alpha, delta, epsilon, gamma, and  $\mu$ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers single monoclonal antibodies (including agonist and antagonist antibodies), antibody compositions with polyepitopic specificity, as well as antibody fragments (e.g., Fab, F(ab')<sub>2</sub>, scFv and Fv), so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler & Milstein, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567 (Cabilly *et al.*)).

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity, *e.g.* binding to and activating mpl (U.S. Patent No. 4,816,567 (Cabilly *et al.*); and Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For

further details see: Jones *et al.*, *Nature*, 321:522-525 (1986); Reichmann *et al.*, *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992).

"Single-chain Fv" or "sFv" antibody fragments comprise the  $V_H$  and  $V_L$  domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a 5 polypeptide linker between the  $V_H$  and  $V_L$  domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain ( $V_H$ ) connected to a light chain variable domain ( $V_L$ ) in the 10 same polypeptide chain ( $V_H$  and  $V_L$ ). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies 15 described in Zapata *et al.* *Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments ( $V_H-C_H1-V_H-C_H1$ ) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

A "variant" antibody, refers herein to a molecule which differs in amino acid sequence from a "parent" antibody amino acid sequence by virtue of addition, deletion and/or substitution of one or more amino acid 20 residue(s) in the parent antibody sequence. In the preferred embodiment, the variant comprises one or more amino acid substitution(s) in one or more hypervariable region(s) of the parent antibody. For example, the variant may comprise at least one, *e.g.* from about one to about ten, and preferably from about two to about five, substitutions in one or more hypervariable regions of the parent antibody. Ordinarily, the variant will have an amino acid sequence having at least 75% amino acid sequence identity with the parent antibody heavy or 25 light chain variable domain sequences, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the parent antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. See Fig. 1. None of N-terminal, C-terminal, or internal extensions, 30 deletions, or insertions into the antibody sequence shall be construed as affecting sequence identity or homology. The variant retains the ability to bind the receptor and preferably has properties which are superior to those of the parent antibody. For example, the variant may have a stronger binding affinity, enhanced ability to activate the receptor, etc. To analyze such properties, one should compare a Fab form of the variant to a Fab form of the parent antibody or a full length form of the variant to a full length form of the parent antibody, for 35 example, since it has been found that the format of the antibody impacts its activity in the biological activity assays disclosed herein. The variant antibody of particular interest herein is one which displays at least about 10 fold, preferably at least about 20 fold, and most preferably at least about 50 fold, enhancement in biological activity when compared to the parent antibody.

The "parent" antibody herein is one which is encoded by an amino acid sequence used for the preparation of the variant. Preferably, the parent antibody has a human framework region and has human antibody constant region(s). For example, the parent antibody may be a humanized or human antibody.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The term "epitope tagged" when used herein refers to an antibody fused to an "epitope tag". The epitope tag polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with activity of the antibody. The epitope tag preferably is sufficiently unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least 6 amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field *et al.* *Mol. Cell. Biol.* 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al.*, *Mol. Cell. Biol.* 5(12):3610-3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky *et al.*, *Protein Engineering* 3(6):547-553 (1990)). In certain embodiments, the epitope tag is a "salvage receptor binding epitope". As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, or IgG<sub>4</sub>) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

The terms "*mpl* ligand", "*mpl* ligand polypeptide", "ML", "thrombopoietin" or "TPO" are used interchangeably herein and include any polypeptide that possesses the property of binding to *mpl*, a member of the cytokine receptor superfamily, and having a biological property of *mpl* ligand. An exemplary biological property is the ability to stimulate the incorporation of labeled nucleotides (e.g. <sup>3</sup>H-thymidine) into the DNA of IL-3 dependent Ba/F3 cells transfected with human *mpl*. Another exemplary biological property is the ability to stimulate the incorporation of <sup>35</sup>S into circulating platelets in a mouse platelet rebound assay. This definition encompasses a polypeptide isolated from a *mpl* ligand source such as aplastic porcine plasma described herein or from another source, such as another animal species, including humans, or prepared by recombinant or synthetic methods. Examples include TPO(332) and rhTPO<sub>332</sub>. Also included in this definition is the thrombopoietic ligand described in WO 95/28907 having a molecular weight of about 31,000 daltons (31kd) as determined by SDS gel under reducing conditions and 28,000 daltons (28kd) under non-reducing conditions. The term "TPO" includes variant forms, such as fragments, alleles, isoforms, analogues, chimera thereof and mixtures of these forms. For convenience, all of these ligands will be referred to below simply as "TPO" recognizing that all individual ligands and ligand mixtures are referred to by this term.

Preferably, the TPO is a compound having thrombopoietic activity or being capable of increasing serum platelet counts in a mammal. The TPO is preferably capable of increasing endogenous platelet counts by at least 10%, more preferably by 50%, and most preferably capable of elevating platelet counts in a human to greater than about  $150 \times 10^9$  per liter of blood.

5 The TPO of this invention preferably has at least 70% overall sequence identity with the amino acid sequence of the highly purified substantially homogeneous porcine *mpl* ligand polypeptide and at least 80% sequence identity with the "EPO-domain" of the porcine *mpl* ligand polypeptide. Alternatively, the TPO of this invention may be a mature human *mpl* ligand (hML), or a variant or post-transcriptionally modified form thereof or a protein having about 80% sequence identity with mature human *mpl* ligand. Alternatively, the TPO  
10 may be a fragment, especially an amino-terminus or "EPO-domain" fragment, of the mature human *mpl* ligand. Preferably, the amino terminus fragment retains substantially all of the human ML sequence between the first and fourth cysteine residues but may contain substantial additions, deletions or substitutions outside that region. According to this embodiment, the fragment polypeptide may be represented by the formula:

X-hTPO(7-151)-Y

15 Where hTPO(7-151) represents the human TPO (hML) amino acid sequence from Cys<sup>7</sup> through Cys<sup>151</sup> inclusive; X represents the amino group of Cys<sup>7</sup> or one or more of the amino-terminus amino acid residue(s) of the mature TPO or amino acid residue extensions thereto such as Met, Lys, Tyr or amino acid substitutions thereof such as arginine to lysine or leader sequences containing, for example, proteolytic cleavage sites (e.g. Factor Xa or thrombin); and Y represents the carboxy terminal group of Cys<sup>151</sup> or one or more  
20 carboxy-terminus amino acid residue(s) of the mature TPO or extensions thereto.

A "TPO fragment" means a portion of a naturally occurring mature full length *mpl* ligand or TPO sequence having one or more amino acid residues or carbohydrate units deleted. The deleted amino acid residue(s) may occur anywhere in the peptide including at either the N-terminal or C-terminal end or internally, so long as the fragment shares at least one biological property in common with *mpl* ligand. *Mpl* ligand fragments typically will have a consecutive sequence of at least 10, 15, 20, 25, 30 or 40 amino acid residues that are identical to the sequences of the *mpl* ligand isolated from a mammal including the ligand isolated from aplastic porcine plasma or the human or murine ligand, especially the EPO-domain thereof. Representative examples of N-terminal fragments are TPO(153), hML<sub>153</sub> or TPO(Met<sup>-1</sup> 1-153).

30 The terms "TPO isoform(s)" and "TPO sequence isoform(s)" or the term "derivatives" in association with TPO, etc. as used herein means a biologically active material as defined below having less than 100% sequence identity with the TPO isolated from recombinant cell culture, aplastic porcine plasma or the human *mpl* ligand. Ordinarily, a biologically active *mpl* ligand or TPO isoform will have an amino acid sequence having at least about 70% amino acid sequence identity with the *mpl* ligand/TPO isolated from aplastic porcine plasma or the mature murine, human *mpl* ligand or fragments thereof, preferably at least about 75%, more  
35 preferably at least about 80%, still more preferably at least about 85%, even more preferably at least about 90%, and most preferably at least about 95%.

TPO "analogues" include covalent modification of TPO or *mpl* ligand by linking the TPO polypeptide to one of a variety of nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417;

4,791,192 or 4,179,337. TPO polypeptides covalently linked to the forgoing polymers are referred to herein as pegylated TPO.

Still other preferred TPO polypeptides of this invention include *mpl* ligand sequence variants and chimeras. Ordinarily, preferred *mpl* ligand sequence variants and chimeras are biologically active *mpl* ligand variants that have an amino acid sequence having at least 90% amino acid sequence identity with the human *mpl* ligand and most preferably at least 95%. An exemplary preferred *mpl* ligand variant is a N-terminal domain hML variant (referred to as the "EPO-domain" because of its sequence homology to erythropoietin). The preferred hML EPO-domain comprises about the first 153 amino acid residues of mature hML and is referred to as hML<sub>153</sub>. An optionally preferred hML sequence variant comprises one in which one or more of the basic or dibasic amino acid residue(s) in the C-terminal domain is substituted with a non-basic amino acid residue(s) (e.g., hydrophobic, neutral, acidic, aromatic, Gly, Pro and the like). A preferred hML C-terminal domain sequence variant comprises one in which Arg residues 153 and 154 are replaced with Ala residues. This variant is referred to as hML<sub>332</sub>(R153A, R154A).

A preferred chimera is a fusion between *mpl* ligand or fragment (defined below) thereof with a heterologous polypeptide or fragment thereof. For example, hML<sub>153</sub> may be fused to an IgG fragment to improve serum half-life or to IL-3, G-CSF or EPO to produce a molecule with enhanced thrombopoietic or chimeric hematopoietic activity.

Other preferred *mpl* ligand fragments have a Met preceding the amino terminus Ser (e.g. Met<sup>-1</sup>TPO<sub>153</sub>). This is preferred when, for example, the protein is expressed directly in a microorganism such as *E. coli*. Optionally, these *mpl* ligand fragments may contain amino acid substitutions to facilitate derivitization. For example, Arg<sub>153</sub> or other residues of the carbohydrate domain may be substituted with Lys to create additional sites to add polyethylene glycol. Preferred *mpl* ligand fragments according to this option include Met<sup>-1</sup>TPO(1-X) where X is about 153, 164, 191, 199, 205, 207, 217, 229, or 245 for the sequence of residues 1-X. Other preferred *mpl* ligand fragments include those produced as a result of chemical or enzymatic hydrolysis or digestion of the purified ligand.

"Essentially pure" protein means a composition purified to remove contaminating proteins and other cellular components, preferably comprising at least about 90% by weight of the protein, based on total weight of the composition, more preferably at least about 95% by weight. "Essentially homogeneous" protein means a composition comprising at least about 99% by weight of protein, based on total weight of the composition.

## 30        II. Preferred Embodiments of the Invention

In one embodiment, preferred antibodies of this invention are substantially homogeneous antibodies and variants thereof, referred to as agonist antibodies (aAb), that possess the property of binding to c-*mpl*, a member of the hematopoietic growth factor receptor superfamily, and transducing a survival, proliferation, maturation and/or differentiation signal. Such signal transduction may be determined by measuring stimulation of incorporation of labeled nucleotides (<sup>3</sup>H-thymidine) into the DNA of IL-3 dependent Ba/F3 cells transfected with human *mpl* P, or with a CMK Assay measuring Induction of the platelet antigen GPIIbIIIa expression. Signal transduction may also be determined by KIRA ELISA by measuring phosphorylation of the c-*mpl*-Rse.gD chimeric receptor, in a c-*mpl*/Mab HU-03 cell proliferation assay or in a liquid suspension megakaryocytopoiesis assay.

Preferred c-*mpl* agonist antibodies of this invention are also capable of inducing or causing survival, proliferation, maturation or differentiation of CD34+ cells into the platelet producing form at a concentration equal to or not less than 2 orders of magnitude (100-fold) below that of thrombopoietin on a weight basis.

More preferred c-*mpl* aAb(s) are substantially purified aAb(s) having hematopoietic, especially megakaryocytopoietic or thrombocytopoietic activity - namely, being capable of stimulating proliferation, maturation and/or differentiation of immature megakaryocytes or their predecessors into the mature platelet-producing form that demonstrate a biological activity equal to or within 2 orders of magnitude of that of rhTPO on a weight basis. Most preferred aAb(s) of this invention are human aAb(s) including full length antibodies having an intact human Fc region and including fragments thereof having hematopoietic, megakaryocytopoietic or thrombopoietic activity. Exemplary fragments having the above described biological activity include; Fv, scFv, F(ab'), F(ab')2.

Preferred scFv fragments denominated 10F6, 5E5, 10D10, 12B5, 12D5 and 12E10 having sequences for CDRs and Framework regions provided in Figure 1. Alternatively, the above enumerated scFvs are affinity matured by mutating 1-3 amino acid residues in one or more of the CDRs or in the framework regions between the CDRs.

The framework regions may be derived from a "consensus sequence" (i.e. the most common amino acids of a class, subclass or subgroup of heavy or light chains of human immunoglobulins) or may be derived from an individual human antibody framework region or from a combination of different framework region sequences. Many human antibody framework region sequences are compiled in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), pages 647-669), for example.

A suitable method for purifying *mpl* antibodies comprises contacting an antibody source containing the *mpl* antibody molecules with an immobilized receptor polypeptide, specifically *mpl* or a *mpl* fusion polypeptide, under conditions whereby the *mpl* antibody molecules to be purified are selectively adsorbed onto the immobilized receptor polypeptide, washing the immobilized support to remove non-adsorbed material, and eluting the molecules to be purified from the immobilized receptor polypeptide with an elution buffer. The source containing the *mpl* antibody may be a library of antibodies having different binding epitopes and the receptor may be immobilized on a plate, tube, particle or other suitable surface using known methods.

Alternatively, the source containing the antibody is recombinant cell culture where the concentration of antibody in either the culture medium or in cell lysates is generally higher than in plasma or other natural sources. The preferred purification method to provide substantially homogeneous antibody comprises: removing particulate debris, either host cells or lysed fragments by, for example, centrifugation or ultrafiltration; optionally, protein may be concentrated with a commercially available protein concentration filter; followed by separating the antibody from other impurities by one or more steps selected from; immunoaffinity, ion-exchange (e.g., DEAE or matrices containing carboxymethyl or sulfopropyl groups), Blue-SEPHAROSE, CM Blue-SEPHAROSE, MONO-Q, MONO-S, lentil lectin-SEPHAROSE, WGA-SEPHAROSE, Con A-SEPHAROSE, Ether TOYPEARL, Butyl TOYPEARL, Phenyl TOYPEARL, protein A SEPHAROSE, SDS-PAGE, reverse phase HPLC (e.g., silica gel with appended aliphatic groups) or SEPHADEX molecular sieve or size exclusion chromatography, and ethanol or ammonium sulfate

precipitation. A protease inhibitor such as methylsulfonylfluoride (PMSF) may be included in any of the foregoing steps to inhibit proteolysis.

Preferably, the isolated antibody is monoclonal (Kohler and Milstein, *Nature*, 256:495-497 (1975); Campbell, *Laboratory Techniques in Biochemistry and Molecular Biology*, Burdon *et al.*, Eds, Volume 13, 5 Elsevier Science Publishers, Amsterdam (1985); and Huse *et al.*, *Science*, 246:1275-1281 (1989)). A preferred *mpl* antibody is one that binds to *mpl* receptor with an affinity of at least about 10<sup>6</sup> l/mole. More preferably the antibody binds with an affinity of at least about 10<sup>7</sup> l/mole or even at least 10<sup>9</sup> l/mole. Most preferably, the antibody is raised against a *mpl* receptor having one of the above described effector functions. The isolated antibody capable of binding to the *mpl* receptor may optionally be fused to a second polypeptide and the 10 antibody or fusion thereof may be used to isolate and purify *mpl* from a source as described above for immobilized *mpl* polypeptide. In a further preferred aspect of this embodiment, the invention provides a method for detecting the *mpl* ligand *in vitro* or *in vivo* comprising contacting the antibody with a sample, especially a serum sample, suspected of containing the ligand and detecting if binding has occurred.

The invention also provides an isolated nucleic acid molecule encoding the *mpl* antibody or fragments 15 thereof, which nucleic acid molecule may be labeled or unlabeled with a detectable moiety, and a nucleic acid molecule having a sequence that is complementary to, or hybridizes under stringent or moderately stringent conditions with, a nucleic acid molecule having a sequence encoding a *mpl* antibody. A preferred *mpl* antibody nucleic acid is RNA or DNA that encodes a biologically active human antibody.

In a further preferred embodiment of this invention, the nucleic acid molecule is cDNA encoding the 20 *mpl* antibody and further comprises a replicable vector in which the cDNA is operably linked to control sequences recognized by a host transformed with the vector. This aspect further includes host cells transformed with the vector and a method of using the cDNA to effect production of antibody, comprising expressing the cDNA encoding the antibody in a culture of the transformed host cells and recovering the antibody from the host cell culture. The antibody prepared in this manner is preferably substantially homogeneous human 25 antibody. A preferred host cell for producing the antibody is Chinese hamster ovary (CHO) cells. An alternative preferred host cell is *E. coli*.

The invention further includes a preferred method for treating a mammal having an immunological or hematopoietic disorder, especially thrombocytopenia comprising administering a therapeutically effective amount of a *mpl* agonist or antagonist antibody to the mammal. Optionally, the antibody is administered in 30 combination with a cytokine, especially a colony stimulating factor or interleukin. Preferred colony stimulating factors or interleukins include; kit-ligand, LIF, G-CSF, GM-CSF, M-CSF, EPO, IL-1, IL-2, IL-3, IL-5, IL-6, IL-7, IL-8, IL-9 or IL-11. Alternatively, the antibody is administered in combination with an Insulin-like growth factor (e.g., IGF-1) or a tumor necrosis factor (e.g., lymphotoxin (LT)).

### III. Methods of Making

35 Nucleic acid encoding the agonist and/or antagonist antibodies of the invention can be prepared from a library of single chain antibodies displayed on a bacteriophage. The preparation of such a library is well known to one of skill in this art. Suitable libraries may be prepared by the methods described in WO 92/01047, WO 92/20791, WO 93/06213, WO 93/11236, WO 93/19172, WO 95/01438 and WO 95/15388. In a preferred embodiment, a library of single chain antibodies (scFv) may be generated from a diverse population of human

B-cells from human donors. mRNA corresponding to the VH and VL antibody chains is isolated and purified using standard techniques and reverse transcribed to generate a population of cDNA. After PCR amplification, DNA coding for single chain antibodies is assembled using a linker, such as Gly<sub>4</sub>Ser, and cloned into suitable expression vectors. A phage library is then prepared in which the population of single chain antibodies is displayed on the surface of the phage. Suitable methods for preparing phage libraries have been reviewed and are described in Winter *et. al.*, *Annu. Rev. Immunol.*, 1994, 12:433-55; Soderlind *et. al.*, *Immunological Reviews*, 1992, 130:109-123; Hoogenboom, *Tibtech* February 1997, Vol. 15; Neri *et. al.*, *Cell Biophysics*, 1995, 27:47-61, and the references described therein.

The antibodies of the invention having agonist or antagonist properties may be selected by immobilizing a receptor and then panning a library of human scFv prepared as described above using the immobilized receptor to bind antibody. Griffiths *et. al.*, *EMBO-J*, 1993, 12:725-734. The specificity and activity of specific clones can be assessed using known assays. Griffiths *et. al.*; Clarkson *et. al.*, *Nature*, 1991, 352:642-648. After a first panning step, one obtains a library of phage containing a plurality of different single chain antibodies displayed on phage having improved binding to the receptor. Subsequent panning steps provide additional libraries with higher binding affinities. When avidity effects are a problem, monovalent phage display libraries may be used in which less than 20%, preferably less than 10%, and more preferably less than 1% of the phage display more than one copy of an antibody on the surface of the phage. Monovalent display can be accomplished with the use of phagemid and helper phage as described, for example, in Lowman *et. al.*, *Methods: A Companion to Methods in Enzymology*, 1991, 3(3):205-216. A preferred phage is M13 and display is preferably as a fusion protein with coat protein 3 as described in Lowman *et. al.*, *supra*. Other suitable phage include f1 and fd filamentous phage. Fusion protein display with other virus coat proteins is also known and may be used in this invention. See U.S. 5,223,409.

Amino acid sequence variants of the antibody are prepared by introducing appropriate nucleotide changes into the antibody DNA, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibodies of the examples herein. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the humanized or variant antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells *Science*, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the receptor. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed antibody variants are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue or the antibody fused to an epitope tag. Other insertional variants of the antibody molecule 5 include the fusion to the N- or C-terminus of the antibody of an enzyme or a polypeptide which increases the serum half-life of the antibody.

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antibody molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also 10 contemplated. Conservative substitutions are shown in Table 2 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 2, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 2

15

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity

of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophilic: cys, ser, thr;
- 5 (3) acidic: asp, glu;
- (4) basic: asn, gln, his, lys, arg;
- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another  
10 class.

Any cysteine residue not involved in maintaining the proper conformation of the humanized or variant antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

15 A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants is affinity maturation using phage using methods known in the art. Briefly, several hypervariable region sites (e.g. 3-7 sites) are  
20 mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identified hypervariable region residues contributing  
25 significantly to antigen binding. Alternatively, or in addition, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and receptor. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

30 Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

35 Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylglucosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

5 Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.

10 Preferably, the antibodies are prepared by standard recombinant procedures which involve production of the antibodies by culturing cells transfected to express antibody nucleic acid (typically by transforming the cells with an expression vector) and recovering the antibody from the cells of cell culture.

15 The nucleic acid (e.g., cDNA or genomic DNA) encoding *mpl* antibody selected as described above is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on (1) whether it is to be used for DNA amplification or for DNA expression, (2) the size of the nucleic acid to be inserted into the vector, and (3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell with which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin 20 of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal Sequence Component

25 The *mpl* antibody of this invention may be expressed not only directly, but also as a fusion with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the *mpl* antibody DNA that is inserted into the vector. The heterologous signal sequence selected should be one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For prokaryotic host cells a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native 30 signal sequence may be substituted by, e.g., the yeast invertase, alpha factor, or acid phosphatase leaders, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native signal sequence (i.e., the *mpl* ligand presequence that normally directs secretion of *mpl* ligand from its native mammalian cells *in vivo*) is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from other 35 *mpl* ligand polypeptides or from the same *mpl* ligand from a different animal species, signal sequences from a *mpl* ligand, and signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2  $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

5 Most expression vectors are "shuttle" vectors, *i.e.*, they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

10 DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a 15 sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of antibody DNA. However, the recovery of genomic DNA encoding antibody is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the antibody DNA.

15 (iii) Selection Gene Component

20 Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) 25 supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

30 One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al.*, *J. Molec. Appl. Genet.*, 1:327 (1982)) mycophenolic acid (Mulligan *et al.*, *Science*, 209:1422 (1980)) or hygromycin Sugden *et al.*, *Mol. Cell. Biol.*, 5:410-413 (1985)). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

35 Examples of other suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the antibody nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that

encodes antibody. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of antibody are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the 5 transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980). The transformed cells are then exposed to increased levels of Mtx. This leads to the synthesis 10 of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding antibody. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with 15 DNA sequences encoding antibody, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3 phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, *Nature*, 282:39 (1979); Kingsman *et al.*, *Gene*, 7:141 (1979); or Tschemper *et al.*, *Gene*, 20 10:157 (1980)). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 85:12 (1977)). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC No. 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

25 (iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the antibody nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the antibody nucleic acid sequence, to which they are 30 operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to antibody encoding DNA by removing the promoter from the source DNA by restriction 35 enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native antibody promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the antibody DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed antibody as compared to the native promoter.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems (Chang *et al.*, *Nature*, 275:615 (1978); and Goeddel *et al.*, *Nature*, 281:544 (1979)), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, *Nucleic Acids Res.*, 8:4057 (1980) and EP 36,776) and hybrid promoters such as the tac promoter (deBoer *et al.*, *Proc. Natl. Acad. Sci. USA*, 80:21-25 (1983)).

5 However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding antibody (Siebenlist *et al.*, *Cell*, 20:269 (1980)) using linkers or adapters to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding antibody polypeptide.

10 Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into 15 eukaryotic expression vectors.

15 Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, *J. Biol. Chem.*, 255:2073 (1980)) or other glycolytic enzymes (Hess *et al.*, *J. Adv. Enzyme Reg.*, 7:149 (1968); and Holland, *Biochemistry*, 17:4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, 20 glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

25 Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytchrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and 20 promoters for use in yeast expression are further described in Hitzeman *et al.*, EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

30 Antibody transcription from vectors in mammalian host cells may be controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock 35 promoters, and from the promoter normally associated with the antibody sequence, provided such promoters are compatible with the host cell systems.

35 The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, *Nature*, 273:113 (1978); Mulligan and Berg, *Science*, 209:1422-1427 (1980); Pavlakis *et al.*, *Proc. Natl. Acad. Sci. USA*, 78:7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a *Hind*III E restriction fragment. Greenaway *et al.*, *Gene*, 18:355-360 (1982). A system for expressing DNA in mammalian hosts

using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Gray *et al.*, *Nature*, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes *et al.*, *Nature*, 297:598-601 (1982) on expression of human  $\beta$ -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, *Proc. Natl. Acad. Sci. USA*, 79:5166-5170 (1982) on expression of the human interferon  $\beta$ 1 gene in cultured mouse and rabbit cells; and Gorman *et al.*, *Proc. Natl. Acad. Sci. USA*, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

10 (v) Enhancer Element Component

Transcription of a DNA encoding the antibody of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5 (Laiminset *et al.*, *Proc. Natl. Acad. Sci. USA*, 78:993 (1981)) and 3 (Lusky *et al.*, *Mol. Cell Bio.*, 3:1108 (1983)) to the transcription unit, within an intron (Banerji *et al.*, *Cell*, 33:729 (1983)), as well as within the coding sequence itself (Osborne *et al.*, *Mol. Cell Bio.*, 4:1293 (1984)). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature*, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5 or 3 to the antibody encoding sequence, but is preferably located at a site 5' from the promoter.

25 (vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding antibody.

30 (vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC No. 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, *Nucleic Acids Res.*, 9:309 (1981) or by the method of Maxam *et al.*, *Methods in Enzymology*, 65:499 (1980).

(viii) Transient Expression Vectors

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding the antibody polypeptide. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Sambrook *et al.*, *supra*, pp. 16.17 - 16.22. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogues and variants of antibody polypeptide that have antibody polypeptide biological activity.

10 (ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the antibody in recombinant vertebrate cell culture are described in Gething *et al.*, *Nature*, 293:620-625 (1981); Mantei *et al.*, *Nature*, 281:40-46 (1979); Levinson *et al.*; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression is pRK5 (EP 307,247 U. S. patent no. 5,258,287) or pSVI6B (PCT Publication No. WO 91/08291).

15 Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryotic cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, *Bacilli* such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescans*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC No. 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC No. 31,537), and *E. coli* W3110 (ATCC No. 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, *in vitro* methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

20 In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for antibody encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* (Beach and Nurse, *Nature*, 290:140 (1981); EP 139,383 published 2 May 1985), *Kluyveromyces* hosts (U.S. Patent No. 4,943,529) such as, e.g., *K. lactis* (Louvencourt *et al.*, *J. Bacteriol.*, 737 (1983)), *K. fragilis*, *K. bulgaricus*, *K. thermotolerans*, and *K. marxianus*, *yarrowia* (EP 402,226), *Pichia pastoris* (EP 183,070; Sreekrishna *et al.*, *J. Basic Microbiol.*, 28:265-278 (1988)), *Candida*, *Trichoderma reesia* (EP 244,234), *Neurospora crassa* (Case *et al.*, *Proc. Natl. Acad. Sci. USA*, 76:5259-5263 (1979)), and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium* (WO 91/00357 published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, *Biochem. Biophys. Res. Commun.*, 112:284-289 (1983); Tilburn *et al.*, *Gene*, 26:205-221 (1983); Yelton *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:1470-1474 (1984)) and *A. niger* (Kelly and Hynes, *EMBO J.*, 4:475-479 (1985)).

Suitable host cells for the expression of glycosylated antibody are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any

higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. See, e.g., Luckow *et al.*, *Bio/Technology*, 6:47-55 (1988); Miller *et al.*, *Genetic Engineering*, Setlow *et al.*, eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda *et al.*, *Nature*, 315:592-594 (1985). A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the antibody DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the antibody is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the antibody DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker *et al.*, *J. Mol. Appl. Gen.*, 1:561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. EP 321,196 published 21 June 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, *J. Gen. Virol.*, 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.*, 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, *Annals N.Y. Acad. Sci.*, 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO<sub>4</sub> and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al.*, *supra*, is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw *et al.*, *Gene*, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, *Virology*, 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Patent No. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, *J. Bact.*, 130:946 (1977) and Hsiao *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 76:3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used.

Prokaryotic cells used to produce the antibody polypeptide of this invention are cultured in suitable media as described generally in Sambrook *et al.*, *supra*.

The mammalian host cells used to produce the antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.*, 58:44 (1979), Barnes and Sato, *Anal. Biochem.*, 102:255 (1980), U.S. Patent No. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985; the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin<sup>TM</sup> drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in *in vitro* culture as well as cells that are within a host animal.

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA*, 77:5201-5205 (1980)), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly <sup>32</sup>P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers,

enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

5 Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, 10 luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu *et al.*, *Am. J. Clin. Path.*, 75:734-738 (1980).

Antibody preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly expressed without a secretory signal.

When the antibody is expressed in a recombinant cell other than one of human origin, the antibody is 15 completely free of proteins or polypeptides of human origin. However, it is still usually necessary to purify the antibody from other recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the *mpl* ligand *per se*. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. Alternatively, a 20 commercially available protein concentration filter (e.g., AMICON or Millipore PELLICON ultrafiltration units) may be used. The antibody may then be purified from the soluble protein fraction. The antibody thereafter is purified from contaminant soluble proteins and polypeptides by salting out and exchange or 25 chromatographic procedures employing various gel matrices. These matrices include; acrylamide, agarose, dextran, cellulose and others common to protein purification. Exemplary chromatography procedures suitable for protein purification include immunoaffinity, receptor affinity (e.g., *mpl*-IgG or protein A SEPHAROSE), hydrophobic interaction chromatography (HIC) (e.g., ether, butyl, or phenyl Toyopearl), lectin chromatography (e.g., Con A-SEPHAROSE, lentil-lectin-SEPHAROSE), size exclusion (e.g., SEPHADEX G-75), cation- and 30 anion-exchange columns (e.g., DEAE or carboxymethyl- and sulfopropyl-cellulose), and reverse-phase high performance liquid chromatography (RP-HPLC) (see e.g., Urdal *et al.*, *J. Chromatog.*, 296:171 (1984) where two sequential RP-HPLC steps are used to purify recombinant human IL-2). Other purification steps optionally include; ethanol precipitation; ammonium sulfate precipitation; chromatofocusing; preparative SDS-PAGE, and the like.

Antibody variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a an antibody fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, 35 facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion polypeptide. Immunoaffinity columns such as a rabbit polyclonal anti-antibody column can be employed to absorb the antibody variant by binding it to at least one remaining immune epitope. Alternatively, the antibody may be purified by affinity chromatography using a purified receptor-IgG coupled to a (preferably) immobilized resin such as AFFI-Gel 10 (Bio-Rad, Richmond, CA) or the like, by means well

known in the art. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native the antibody may require modification to account for changes in the character of the antibody or its variants 5 upon expression in recombinant cell culture.

In a most preferred embodiment of the invention, the antibodies are agonist antibodies (aAb). By "agonist antibody" is meant an antibody which is able to bind to and to activate, a particular hematopoietic growth factor receptor. For example, the agonist may bind to the extracellular domain of the receptor and thereby cause differentiation and proliferation of megakaryocyte colonies in semisolid cultures and single 10 megakaryocytes in liquid suspension cultures and platelet production *in vitro* and/or *in vivo*. The agonist antibodies are preferably against epitopes within the extracellular domain of the receptor. Accordingly, the antibody preferably binds to substantially the same epitope as the 12E10, 12B5, 10F6, and 12D5 monoclonal antibodies specifically disclosed herein. Most preferably, the antibody will also have substantially the same or greater antigen binding affinity as the monoclonal antibodies disclosed herein. To determine whether a 15 monoclonal antibody has the same specificity as an antibody specifically disclosed, one can, for example, use a competitive ELISA binding assay.

DNA encoding the monoclonal antibodies useful in the method of the invention is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of human antibodies). The phage of the invention 20 serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

#### IV. Utility

25 The antibodies disclosed herein are useful for *in vitro* diagnostic assays for activating the receptor of interest. This is useful in order to study the role of the receptor in megakaryocyte growth and/or differentiation and platelet production.

The biologically active *cmpl* agonist antibody capable of stimulating either proliferation, differentiation or maturation and/or modulation (either stimulation or inhibition) of apoptosis of hematopoietic 30 cells may be used in a sterile pharmaceutical preparation or formulation to stimulate megakaryocytopoietic or thrombopoietic activity in patients suffering from thrombocytopenia due to impaired production, sequestration, or increased destruction of platelets. Thrombocytopenia-associated bone marrow hypoplasia (e.g., aplastic anemia following chemotherapy or bone marrow transplant) may be effectively treated with the aAb compounds of this invention as well as disorders such as disseminated intravascular coagulation (DIC), immune 35 thrombocytopenia (including HIV-induced ITP and non HIV-induced ITP), chronic idiopathic thrombocytopenia, congenital thrombocytopenia, myelodysplasia, and thrombotic thrombocytopenia.

Preferred uses of the megakaryocytopoietic or thrombocytopoietic biologically active *cmpl* agonist antibody of this invention are in: myelotoxic chemotherapy for treatment of leukemia or solid tumors,

myeloablative chemotherapy for autologous or allogeneic bone marrow transplant, myelodysplasia, idiopathic aplastic anemia, congenital thrombocytopenia, and immune thrombocytopenia.

The biologically active *c-mpl* agonist antibody of the instant invention may be employed alone or in combination with other cytokines, hematopoietins, interleukins, growth factors, or antibodies in the treatment of the above-identified disorders and conditions. Thus, the instant compounds may be employed in combination with other protein or peptide having hematopoietic activity including G-CSF, GM-CSF, LIF, M-CSF, IL-1, IL-3, erythropoietin (EPO), kit ligand, IL-6, and IL-11.

The biologically active *c-mpl* agonist antibody of the instant invention may be used in the same way and for the same indications as thrombopoietin (TPO). Some forms of the aAb have a longer half-life than native or pegylated TPO and thus are used in indications where a longer half-life are indicated.

When used for *in vivo* administration, the antibody formulation must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The antibody ordinarily will be stored in lyophilized form or in solution.

Therapeutic antibody compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of antibody administration is in accord with known methods, *e.g.*, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, intrathecal, inhalation or intralesional routes, or by sustained release systems as noted below. The antibody is preferably administered continuously by infusion or by bolus injection.

An effective amount of antibody to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. Typically, the clinician will administer antibody until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

The antibodies of the invention may be prepared in a mixture with a pharmaceutically acceptable carrier. This therapeutic composition can be administered intravenously or through the nose or lung, preferably as a liquid or powder aerosol (lyophilized). The composition may also be administered parenterally or subcutaneously as desired. When administered systematically, the therapeutic composition should be sterile, pyrogen-free and in a parenterally acceptable solution having due regard for pH, isotonicity, and stability.

These conditions are known to those skilled in the art. Briefly, dosage formulations of the compounds of the present invention are prepared for storage or administration by mixing the compound having the desired degree of purity with physiologically acceptable carriers, excipients, or stabilizers. Such materials are non-toxic to the recipients at the dosages and concentrations employed, and include buffers such as TRIS HCl, phosphate, citrate, acetate and other organic acid salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) peptides such as polyarginine, proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidinone; amino acids such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol

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or sorbitol; counterions such as sodium and/or nonionic surfactants such as TWEEN, PLURONICS or polyethyleneglycol.

Sterile compositions for injection can be formulated according to conventional pharmaceutical practice. For example, dissolution or suspension of the active compound in a vehicle such as water or naturally occurring vegetable oil like sesame, peanut, or cottonseed oil or a synthetic fatty vehicle like ethyl oleate or the like may be desired. Buffers, preservatives, antioxidants and the like can be incorporated according to accepted pharmaceutical practice.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, *J. Biomed. Mater. Res.*, 15:167-277 (1981) and Langer, *Chem. Tech.*, 12:98-105 (1982) or poly(vinylalcohol)), polylactides (U.S. Patent No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, *Biopolymers*, 22:547-556 (1983)), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the LUPRON Depot<sup>TM</sup> (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through disulfide interchange, stabilization may be achieved by modifying sulphydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release compositions also include liposomally entrapped TPO. Liposomes containing TPO are prepared by methods known *per se*: DE 3,218,121; Epstein *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:3688-3692 (1985); Hwang *et al.*, *Proc. Natl. Acad. Sci. USA*, 77:4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Patent Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal therapy.

The dosage of the antibody will be determined by the attending physician taking into consideration various factors known to modify the action of drugs including severity and type of disease, body weight, sex, diet, time and route of administration, other medications and other relevant clinical factors. Therapeutically effective dosages may be determined by either *in vitro* or *in vivo* methods.

An effective amount of the agonist antibody to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 µg/kg to up to 1000

mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer the molecule until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

Depending on the type and severity of the disease, from about 0.001 mg/kg to about 1000 mg/kg, more 5 preferably about 0.01 mg to 100 mg/kg, more preferably about 0.010 to 20 mg/kg of the agonist antibody might be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs or the desired improvement in the patient's condition is achieved. However, other dosage regimens may also be useful.

10

#### Examples

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and illustrative examples, make and utilize the present invention to the fullest extent. The following working examples therefore specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way of the remainder of the disclosure.

15

#### Example 1

##### Assays

The *mpl* agonist antibody assays were conducted essentially as described in WO 95/18858.

(a) Ba/F3 cell proliferation assay

The Ba/F3-*mpl* cell line was established (F. de Sauvage *et al.*, *Nature*, 369:533 (1994)) by introduction 20 of the cDNA encoding the entire *mpl* receptor into the IL-3 dependent murine lymphoblastoid cell line Ba/F3. Stimulation of proliferation of Ba/F3-*mpl* cells in response to various concentrations of antibodies or TPO was measured by the amount of incorporation of  $^3$ H-thymidine as previously described (F. de Sauvage *et al.*, *supra*).

(b) CMK Assay for Induction of Platelet Antigen GPII<sub>b</sub>III<sub>a</sub> Expression

CMK cells are maintained in RMPI 1640 medium (Sigma) supplemented with 10% fetal bovine serum 25 and 10mM glutamine. In preparation for the assay, the cells are harvested, washed and resuspended at  $5 \times 10^5$  cells/ml in serum-free GIF medium supplemented with 5mg/l bovine insulin, 10mg/l apo-transferrin, 1 X trace elements. In a 96-well flat-bottom plate, the TPO standard or experimental agonist antibody samples are added to each well at appropriate dilutions in 100 ml volumes. 100 ml of the CMK cell suspension is added to each 30 well and the plates are incubated at 37°C, in a 5% CO<sub>2</sub> incubator for 48 hours. After incubation, the plates are spun at 1000rpm at 4°C for five minutes. Supernatants are discarded and 100 ml of the FITC-conjugated GPII<sub>b</sub>III<sub>a</sub> monoclonal 2D2 antibody is added to each well. Following incubation at 4°C for 1 hour, plates are spun again at 1000rpm for five minutes. The supernatants containing unbound antibody are discarded and 200 35 ml of 0.1% BSA-PBS wash is added to each well. The 0.1% BSA-PBS wash step is repeated three times. Cells are then analyzed on a FASCAN using standard one parameter analysis measuring relative fluorescence intensity.

(c) KIRA ELISA for Measuring Phosphorylation of the *mpl*-Rse.gD Chimeric Receptor

The human *mpl* receptor has been disclosed by Vigon *et al.*, *PNAS, USA* 89:5640-5644 (1992). A chimeric receptor comprising the extracellular domain (ECD) of the *mpl* receptor and the transmembrane and

intracellular domain (ICD) of *Rse* (Mark *et al.*, *J. of Biol. Chem.* 269(14):10720-10728 (1994)) with a carboxyl-terminal flag polypeptide (*i.e.* *Rse.gD*) was made for use in the KIRA ELISA described herein.

(i) Capture agent preparation

5 Monoclonal anti-gD (clone 5B6) was produced against a peptide from Herpes simplex virus glycoprotein D (Paborsky *et al.*, *Protein Engineering* 3(6):547-553 (1990)). The purified stock preparation was adjusted to 3.0mg/ml in phosphate buffered saline (PBS), pH 7.4 and 1.0ml aliquots were stored at -20° C.

(ii) Anti-phosphotyrosine antibody preparation

10 Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from UBI (Lake Placid, NY) and biotinylated using long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics, Cleveland, OH).

(iii) Ligand

The *mpl* ligand was prepared by the recombinant techniques described herein. The purified *mpl* ligand was stored at 4 °C. as a stock solution.

15 KIRA ELISA results for agonist antibodies of the invention are shown in Fig. 9. This assay indicates that the antibodies of the invention activate the *mpl* receptor to a degree similar to the cognate ligand TPO.

(d) TPO receptor-binding inhibition assay

20 NUNC 96-well immunoplates were coated with 50 µl of rabbit anti-human IgG Fc (Jackson Labs) at 2 µg/ml in carbonate buffer (pH9.6) overnight at 4°C. After blocking with ELISA buffer (PBS, 1 % BSA, 0.2 % TWEEN 20), the plates were incubated for 2 hr with conditioned media from *mpl*-Ig-transfected 293 cells. Plates were washed, and 2.5 ng/ml biotinylated TPO was added in the presence or absence of various concentrations of antibodies. After incubation for 1 hr and washing, the amount of TPO bound was detected by incubation with streptAvidin-HRP (Sigma) followed by TMB peroxidase substrate (Kirkegaard & Perry). All dilutions were performed in ELISA buffer, and all incubations were at room temperature. Color development was quenched with H<sub>3</sub>PO<sub>4</sub> and absorbance was read at 450-650 nm.

25 (e) HU-03 cell proliferation assay

The HU-01 cell line (D. Morgan, Hahnemann University) is derived from a patient with acute megakaryoblastic leukemia and is dependent on granulocyte-macrophage colony stimulating factor (GM-CSF) for growth. The HU-03 cell line used here was derived from HU-01 cells by adaptation to growth in rhTPO rather than GM-CSF.

30 HU-03 cells were maintained in RPMI 1640 supplemented with 2 % heat-inactivated human male serum and 5 ng/ml rhTPO. Before assay, cells were starved by removing TPO, decreasing serum concentration to 1 %, and adjusting the concentration of cells to 2.5 x 10<sup>5</sup> cells/ml, followed by incubation for 16 hr. Cells were then washed and seeded into 96-well plates at a density of 5 x 10<sup>4</sup> cells per well in medium containing TPO or antibodies at various concentrations. Quadruplicate assays were performed. 1 µCi 3H-thymidine was added to each well before incubation for 24 hr. Cells were collected with a Packard cell harvester and incorporation of <sup>3</sup>H-thymidine was measured with a Top Count Counter (Packard).

(f) Liquid suspension megakaryocytopoiesis assay

The effect of Mpl agonist antibodies on human megakaryocytopoiesis was determined using a modification of the liquid suspension assay previously described (Grant *et al.*, *Blood* 69:1334-1339 (1997)).

Buffy coats were collected from human umbilical cord blood and cells washed in phosphate-buffered saline (PBS) by centrifugation at 120 g for 15 min at room temperature to remove platelet-rich plasma. Cell pellets were resuspended in Iscove's modified Dulbecco's medium (IMDM, GIBCO) (supplemented with 100 units per ml penicillin and streptomycin), layered onto 60% percoll (density = 1.077 gm/ml, Pharmacia), and centrifuged 5 at 800 g for 20 min at room temperature. The light-density mononuclear cells were collected from the interface and washed twice with IMDM. Cells were seeded at  $1 \times 10^6$  cells per ml in IMDM supplemented with 30% fetal bovine serum (FBS), 100 units per ml penicillin and streptomycin, and 20  $\mu$ M 2-mercaptopethanol, into 24-well tissue culture plates (COSTAR). Serial dilutions of thrombopoietin (TPO) or the Fab'2 forms of antibody 12B5 or antibody 12D5 were added to quadruplicate wells; control wells contained no 10 additional supplements. Final volumes were 1 ml per well. The cultures were grown in a humidified incubator at 37 °C in 5% CO<sub>2</sub> for 14 days. Megakaryocytopoiesis was quantified using radiolabelled murine monoclonal antibody HP1-1D (provided by W. L. Nichols, Mayo Clinic) which has been shown to be specific for the human megakaryocyte glycoprotein IIb/IIIa (Grant *et al.*, *supra*). Cells were harvested from the tissue culture plates, washed twice with assay buffer (20% FBS, 0.002% EDTA in PBS), and resuspended in 100  $\mu$ l assay 15 buffer containing 20 ng iodinated HP1-1D (approximately 100,000 cpm). After incubation at room temperature for 1 hr, the cells were washed twice with assay buffer and the cell pellets counted with a gamma counter.

FBS used in this assay was treated with Dextran T40 at 1 mg/ml and charcoal at 10 mg/ml for 30 min, centrifuged, decanted, filter sterilized and heat inactivated at 56 °C for 30 min.

20 (g) TPO-antibody competitive binding assays for HU-03 cells and human platelets

HU-03 cells were cultured as described above. Platelet rich plasma (PRP) was prepared by centrifugation of citrated whole blood at 400 g's for 5 minutes. Binding studies were conducted within three hours of collection. <sup>125</sup>I-TPO was prepared by indirect iodination (Fielder, P. J., Hass, P., Nagel, M., Stefanich, E., Widmer, R., Bennett, G. L., Keller, G., de Savage, F. J., and Eaton, D. 1997. Human platelets as 25 a model for the binding and degradation of thrombopoietin. *Blood* 89: 2782-2788) and yielded a specific activity of 15-50  $\mu$ Ci/ $\mu$ g protein.

In a volume of 110 microliters containing 100pM iodinated TPO,  $2 \times 10^6$  washed HU-03 cells in Hank's Balanced Salt Solution, 5 mg/ml bovine serum albumin (HBSSB), or  $4 \times 10^7$  platelets in plasma, were 30 incubated at 37°C for 30 minutes with varying concentrations of antibody in triplicate. HU-03 cells were agitated during the incubation period to keep them in suspension. The reaction mixture was overlayed on 1 ml 20 % sucrose-HBSSB and microcentrifuged at 13,500 rpm for five minutes. The supernatants were aspirated, tube bottoms containing the cell pellets were cut off, and cell- or platelet-associated radioactivity was measured with an Iso Data Model 120 gamma counter.

35 Results for several agonist antibodies on the invention in this assay are shown in Fig. 10A-F. Longer bars in the graphs indicate greater amounts of bound radiolabeled TPO and less competition by the agonist antibody at a particular concentration.

(h) Affinity determinations.

The receptor-binding affinities of several Fab fragments were calculated (Lofas & Johnsson, 1990) from association and dissociation rate constants measured using a BIACORE surface plasmon resonance system

(Pharmacia Biosensor). A biosensor chip was activated for covalent coupling of gD-mpl receptor using N-ethyl-N'''-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's (Pharmacia Biosensor) instructions. gD-mpl was buffer-exchanged into 10 mM sodium acetate buffer (pH 4.5) and diluted to approximately 30  $\mu$ g/mL. An aliquot (35  $\mu$ L) was injected at a flow rate of 1  $\mu$ L/min to achieve approximately 6400 response units (RU) of coupled protein. Finally, 1M ethanolamine was injected as a blocking agent. For kinetics measurements, 1.5 serial dilutions of Fab were injected in PBS/Tween buffer (0.05% Tween-20 in phosphate buffered saline) at 25°C using a flow rate of 20  $\mu$ L/min. Equilibrium dissociation constants,  $K_d$ 's, from SPR measurements were calculated as  $k_{off}/k_{on}$ . Standard deviations,  $s_{on}$  for  $k_{on}$  and  $s_{off}$  for  $k_{off}$ , were obtained from measurements with >4 protein concentrations ( $k_{on}$ ) or with >7 protein concentrations ( $k_{off}$ ). Dissociation data were fit to a simple AB-->A+B model to obtain  $k_{off}$  +/- s.d. (standard deviation of measurements). Pseudo-first order rate constant (ks) were calculated for each association curve, and plotted as a function of protein concentration to obtain  $k_{on}$  +/- s.e. (standard error of fit). The resulting errors  $e[K]$  in calculated  $K_d$ 's were estimated according to the following formula for propagation of errors:  $e[K] = [(k_{on})^{-2}(s_{off})^2 + (k_{off})^2(k_{on})^{-4}(s_{on})^2]^{1/2}$  where  $s_{off}$  and  $s_{on}$  are the standard errors in  $k_{on}$  and  $k_{off}$ , respectively.

#### Example 2

##### Isolation of Antibodies from the CAT library

For construction of a library of antibodies displayed of a phage see the following references: WO 92/01047, WO 92/20791, WO 93/06213, WO 93/11236, WO 93/19172, WO 95/01438 and WO 95/15388. Briefly, Figures 2 and 3 presents a cartoon of the construction of a phage library containing  $6 \times 10^9$  different clones containing single-chain Fv (scFv) antibodies fused to gene 3 of a phage. Binding selection against an antigen, in this case c-mpl, can be carried out as shown in Fig.4 and described in greater detail below.

###### (a) The Antigen

Human c-mpl was cloned as described by F. de Sauvage *et al.*, *Nature* 369:533 (1994).

###### (b) Phage Selection on immunotubes

NUNC immunotubes were coated with 2 ml of a solution of 10 microg/ml of gD-c-mpl in PBS at 4°C overnight. After rinsing with PBS, tubes were blocked with 3% dry milk in PBS (MPBS) for 2 hr at room temperature. For the first round, 10  $\mu$ l of C.A.T. antibody phage library containing  $\sim 1 \times 10^{12}$  c.f.u. were added to 1 ml MPBS for blocking for 1 hr at room temperature. Blocked phage were added to coated tubes, and binding of phage to antigen allowed to continue for 2 hr at 37°C on a rotating wheel. Tubes were washed 6 times with PBS-TWEEN and 6 times with PBS, and phage were then eluted with 100 mM TEA for 10 min at room temperature, neutralized with 500  $\mu$ l of 1 M TRIS (pH 7.4), and stored on ice until needed. For subsequent rounds, washing was increased to 20 times with PBS-TWEEN, and 20 times with PBS.

Eluted phage were used to infect 5 ml of log phase *E. coli* TG1 cells and plated on 2YT agar supplemented with 2% glucose and 100  $\mu$ g/ml carbenicillin. After overnight growth at 30°C, colonies were scraped into 10 ml 2YT. 50  $\mu$ l of this solution was used to inoculate 25 ml of 2YT with carbenicillin and glucose and incubated, shaking, for two hours at 37°C. Helper phage M13K07 (Pharmacia) were added at an m.o.i. of 10. After adsorption, the cells were pelleted and resuspended in 25 ml of 2YT with carbenicillin (100  $\mu$ g/ml) and kanamycin (50  $\mu$ g/ml) and growth continued at 30°C for 4 hr. *E. coli* were removed from the

phage by centrifugation, and 1 ml of these phage (approx.  $10^{12}$  c.f.u.) were used in subsequent rounds of selection.

(c) Antibody Phage Selection using streptavidin-coated paramagnetic beads

The library was also selected using soluble biotinylated antigen and streptavidin-coated paramagnetic beads (see Fig. 5). gD-c-mpl was biotinylated using IMMUNOPURE NHS-biotin (biotin-N-hydroxy-succinimide, Pierce) according to manufacturer's recommendations.

For the first round of panning, 10  $\mu$ l of the phage library were blocked with 1 ml of MPBST (3% dry milk powder, 1 x PBS, 0.2% TWEEN 20) for 1 hour on a rotating wheel at room temperature. Biotinylated gD-c-mpl was then added to a final concentration of 100 nM, and phage were allowed to bind antigen for 1 hour at 37°C on a rotator. Meanwhile, 300  $\mu$ l of DYNABEADS M-280, coated with streptavidin (DYNAL) were washed 3 times with 1 ml MPBST (using a DYNAL Magnetic Particle Concentrator) and then blocked for 2 hr at 37°C with 1 ml fresh MPBST on a rotator. The beads were collected with the MPC, resuspended in 50  $\mu$ l of MPBST, and added to the phage-plus-antigen solution. Mixing continued on a wheel at room temperature for 15 min. The DYNABEADS and attached phage were then washed a total of 7 times: 3 times with 1 ml PBS-TWEEN, once with MPBS, followed by 3 times with PBS. Phage were eluted from the beads by incubating 5 min at room temperature with 300  $\mu$ l of 100 mM triethylamine. The phage-containing supernatant was removed and neutralized with 150  $\mu$ l of 1M TRIS-HCl (pH 7.4). Neutralized phage were used to infect mid-log TG1 host cells as described above. Plating, induction and harvesting of phage were also as for selection on tubes.

For the second and subsequent rounds of selection on biotinylated gD-c-mpl, 1 ml of harvested phage (approximately  $10^{12}$  cfu) were blocked with 200  $\mu$ l 10% dry milk, 6 X PBS, 0.3% TWEEN 20. Antigen concentration was decreased at each round of selection. In one series the concentrations were: first round, 100 nM; second round, 10 nM; third round, 1 nM. A second panning was performed using: first round 100 nM; second round 100 nM; third round, 50 nM; fourth round, 10 nM; and fifth round, 1 nM. Washing stringency was increased to two cycles of 7 washes for rounds 2, and three cycles for rounds 3 and beyond.

(d) ELISA screening of selected clones

After each round of selection, individual carbenicillin-resistant colonies were screened by ELISA to identify those producing c-mpl-binding phage. Only those clones which were positive in two or more assay formats were further studied. Fig. 6 illustrates the phage ELISA process.

Individual clones were inoculated into 2TY with 2% glucose and 100  $\mu$ g/ml carbenicillin in 96-well tissue culture plates and grown until turbid. Cultures were then infected at an m.o.i. of 10 with M12KO7 helper phage, and infected cells were transferred to 2YT media containing carbenicillin (100  $\mu$ g/ml) and kanamycin (50  $\mu$ g/ml) for growth overnight at 30°C with gentle shaking.

NUNC MAXISORP microtiter plates were coated with 50  $\mu$ l per well of gD-c-mpl, BSA, or gD-gp120, at 2  $\mu$ g/ml in 50 mM carbonate buffer (pH 9.6), at 4°C overnight. After removing antigen, plates were blocked with 3% dry milk in PBS (MPBS) for 2 hours at room temperature.

Phage cultures were centrifuged and 100  $\mu$ l of phage-containing supernatants were blocked with 20  $\mu$ l of 6 x PBS / 18% dry milk for 1 hour at room temperature. Block was removed from titer plates and blocked phage added and allowed to bind for 1 hour at room temperature. After washing, phage were detected with a

1:5000 dilution of horseradish peroxidase-conjugated anti-M13 antibody (Pharmacia) in MPBS followed by 3',3',5',5'-tetramethylbenzidine (TMB). Reactions were stopped by the addition of H<sub>2</sub>SO<sub>4</sub> and readings taken by subtracting the A<sub>405nm</sub> from the A<sub>450nm</sub>.

(e) Soluble scFv ELISA

5 Soluble scFv was induced in the bacterial supernatants of clones by growth in 2YT containing carbenicillin (100 µg/ml) and IPTG (1mM) ON at 30°C. ELISA plates were either coated with gD-c-mpl or, for capture ELISA, with anti-c-myc Mab 9E10. Plates were blocked with 1 x ELISA diluent (PBS supplemented with 0.5% BSA, 0.05% Tween 20, pH 7.4), and soluble scFv was blocked by adding 20 µl of 6 x ELISA dil to 100 µl of supernatant. After binding to antigen coated plates, soluble scFv was detected by adding 50 µl of 1 µg/ml Mab 9E10 per well, followed by horseradish peroxidase-conjugated goat anti-murine Ig, and then TMB as described above. For capture ELISA, soluble scFv was detected by addition of biotinylated c-mpl, followed by streptavidin-peroxidase conjugate and then TMB as above.

The number of clones screened by ELISA from each round, and the number of clones positive by phage ELISA are shown in Table 3.

15 Table 3 - Anti-c-mpl scFv antibodies from CAT library

Clones screened:	1534
Clones positive by ELISA:	361
Clones different by BstNI and sequencing:	24
Clones that express protein well	17
20 clones that are agonists by KIRA:	9
clones that are agonists by BaF3 proliferation assay:	6
clones that are agonists by Hu3 proliferation assay:	4

(f) DNA fingerprinting of clones

The diversity of c-mpl-binding clones was determined by PCR amplifying the scFv insert using 25 primers pUC19R (5 AGC GGA TAA CAA TTT CAC ACA GG 3 ) (SEQ. ID. NO: 54) which anneals upstream of the leader sequence and fdtetseq (5 GTC GTC TTT CCA GAC GGT AGT 3 ) (SEQ. ID. NO: 55) which anneals in the 5' end of gene III, followed by digestion with the frequent-cutting restriction enzyme BstNI (see Fig. 7).

Typical patterns seen after analysis on a 3% agarose gel are shown in Fig. 8A-C.

30 DNA Fingerprinting: Protocol

Mix A: dH2O	67 µl
10 x ampliTaq buffer	10
25 mM MgCl <sub>2</sub>	10
DMSO, 50%	2
35 forward primer	1
Mix B: 2.5 mM dNTPs	8 µl
AMPLITAQ	0.5
reverse primer	1.0

Place 90  $\mu$ l of Mix A in reaction tube  
Inoculate with very small portion of *E.coli* colony using a yellow tip  
Heat in PCR block to 98°C, 3 min. Remove to ice.  
Add 10  $\mu$ l Mix B  
5 Cycle: 95° C, 30 sec, 55°C 30 sec, 72°C 1min 20 sec, for 25 cycles, in Perkin Elmer 2400  
Remove 10  $\mu$ l to run on a 1 % agarose gel, test for a 1 kB band

Make remaining mix to 1 x BstNI reaction buffer  
Add 5 units BstNI  
10 60°C, 2 hours

Electrophorese samples on 3 % METAPHORE agarose gel

(g) Sequencing of clones

The nucleotide sequence of representative clones of each fingerprint were obtained. Colonies were inoculated into 50 ml of LB medium supplemented with 2% glucose and 100  $\mu$ g/ml carbenicillin, and grown overnight at 30°C. DNA was isolated using Qiagen Tip-100s and the manufacturer's protocol and cycle sequenced with fluorescent dideoxy chain terminators (Applied Biosystems). Samples were run on an Applied Biosystems 373A Automated DNA Sequencer and sequences analyzed using the program "Sequencher" (Gene Codes Corporation). The VH and VL genes were assigned to a germline segment using the antibody database, V-BASE.

20 DNA sequence was obtained for 39 clones and resulted in 24 different c-mpl-binding scFvs.

(h) Purification of scFvs with (his)<sub>6</sub>

For protein purification of soluble antibody, *E. coli* strain 33D3 was transformed with phagemid DNA. Five ml of 2YT with carbenicillin and glucose was used to grow overnight cultures at 30°C. 0.2 ml of these cultures were diluted into 200 ml of the same media and grown to an OD<sub>600</sub> of approximately 0.9. The cells 25 were pelleted and resuspended in 250 ml of 2YT containing IPTG (1 mM) and carbenicillin (100  $\mu$ g/ml) and to induce expression and grown for a further 5 hours at 30°C. Cell pellets were harvested and frozen at -20°C.

The antibodies were purified by immobilized metal chelate affinity chromatography (IMAC). Frozen pellets were resuspended in 10 ml of ice-cold shockate buffer (25 mM TRIS-HCl, 1 mM EDTA, 200 mM NaCl, 20 % sucrose, 1 mM PMSF) by shaking on ice for 1 hr. Imidazole was added to 20 mM, and cell debris 30 removed by centrifugation. The supernatants were adjusted to 1mM MgCl<sub>2</sub> and 50 mM phosphate buffer pH 7.5. Ni-NTA agarose resin from Qiagen was used according to the manufacturers instructions. The resin was equilibrated with 50 mM sodium phosphate buffer pH 7.5, 500 mM NaCl, 20 mM imidazole, and the shockate added. Binding occurred in either a batch mode or on a gravity flow column. The resin was then washed twice with 10 bed volumes of equilibration buffer, and twice with buffer containing imidazole increased to 50mM. 35 Elution of proteins was with 50 mM phosphate buffer pH 7.5, 500 mM NaCl and 250 mM imidazole. Excess salt and imidazole was removed on a PD-10 column (Pharmacia), and proteins were concentrated using a Centricom 10 to a volume of about 1 ml.

Concentration was estimated spectrophotometrically assuming an A280 nm of 1.0 = 0.6 mg/ml.

Portions of these protein preparations were submitted for KIRA assay, c-mpl-Ba/F3 cell proliferation assay, and Hu3 cell proliferation assay.

Plasmid DNA for scFv clones 12B5, 12D5, 12E10, 10D10, 10F6 and 5E5 (named pMpl.12B5.scFv.his; pMpl.12D5.scFv.his; pMpl.12E10.scFv.his; pMpl.10D10.scFv.his; pMpl.10F6.scFv.his; and pMpl.5E5.scFv.his, respectively) has been deposited with ATCC, Manassas, Virginia, USA.

5 (i) Reformatting of antibodies to scFv with gD tag, Fab', Fab'2, and full length molecules.

For improved expression of scFv, and for Fab', and Fab'2 forms of antibodies, some of the anti-c-mpl clones were cloned into derivatives of the expression vector pAK19 (Carter *et al.* METHODS: A companion to Methods in Enzymology. 3:183-192 (1991). Expression is under the transcriptional control of the *E. coli* alkaline phosphatase (*phoA*) promoter (Chang, *et al* *Gene* 44:121-125 (1986) which is inducible by phosphate starvation. Each peptide chain is preceded by the *E. coli* enterotoxin II (stII) signal sequence (Picken, *et al.*) to direct secretion to the periplasmic space of *E. coli*. This vector also contains the human  $\kappa_1$   $C_L$  (Palm *et al.* *Infect. Immun.* 42:269-275 (1983)) and the human IgG1  $C_H1$  (Ellison, *et al.*, *Nucleic Acids Res.* 10: 4071-4079 (1982)) constant domains. The  $C_H1$  gene is immediately followed by the bacteriophage  $\lambda$   $t_0$  transcriptional 10 terminator (Scholtissek and Grosse *Nucleic Acids Res.* 15: 3185 (1987)).

15 (j) Fab' and Fab'2 construction

Construction of the Fab' and Fab'2 variants was facilitated by insertion into pAK19 of unique restriction sites at the junctions of the stII and  $V_L$  domain (*Sfi* I), the  $V_L$  and  $C_k$  domains (*Rsr* II), the stII and  $V_H$  domain (*Mlu*I), and the  $V_H$  and  $C_H1$  domains (*Apa* I), using oligonucleotide directed mutagenesis. In order to 20 insure expression of monovalent Fab' molecules, the free cysteine at the 3' end of the  $CH1$  domain was mutated to a threonine, these Fab' molecules thus end in the amino acid sequence thr-ala-ala-pro, rather than thr-cys-ala-ala as in pAK19. This vector for the expression of Fab' molecules is named pXCA730.

Since some of the antibodies derived from the library had light chains which were derived from lambda rather than kappa light chain families, the human  $\lambda$   $C_L$  was subcloned from pB11.2 (Carter, P, Garrard, 25 L., Henner, D. 1991. Methods: A Companion to Methods in Enzymology. 3:183-192) into a derivative of pXCA730 to give vector pXCA970.

For expression of the antibodies as Fab'2 molecules, a vector was constructed which adds the human IgG1 hinge region onto the  $C_H1$  domain of pXCA730. This is followed by the yeast GCN4 leucine zipper domain (Hu, *et al.* *Science* 250:1400-1403 (1990)) for stability. These DNA fragments were constructed using 30 synthesized oligonucleotides and encode the amino acid sequence: cys-pro-pro-cys-ala-pro-glu-leu-leu-gly-gly-arg-met-lys-gln-leu-glu-asp-lys-val-glu-glu-leu-leu-ser-lys-asn-tyr-his-leu-glu-asn-glu-val-ala-arg-leu-lys-leu-val-gly-glu-arg (SEQ. ID. NO: 56). The resultant plasmid is named pXCA740.

The variable domains of the scFvs were amplified and restriction sites added for subcloning into the vectors described above by the PCR technique. Specific oligonucleotides were designed for each  $V_L$  or  $V_H$  35 region as shown below.

12B5, 12D5, and 10D10 Light chain variable domains:

5 primer

GCT TCT GCG GCC ACA CAG GCC TAC GCT GAC ATC GTG ATG ACC C (SEQ. ID. NO: 57)

3 primer

P0979R1

ATG ATG ATG TGC CAC GGT CCG TTT GAT CTC CAG TTC GGT C (SEQ. ID. NO: 58)

12E10 Light chain variable domain:

5 primer

GCT TCT GCG GCC ACA CAG GCC TAC GCT TCC TAT GTG CTG ACT C (SEQ. ID. NO: 59)

5 primer

CCT TCT CTC TTT AGG TTG GCC AAG GAC GGT CAG CTT GGT C (SEQ. ID. NO: 60)

10F6 Light chain variable domain

5 primer

GCT TCT GCG GCC ACA CAG GCC TAC GCT CAG TCT GTG CTG ACT C (SEQ. ID. NO: 61)

10 primer

CCT TCT CTC TTT AGG TTG GCC AAG GAC GGT CAG CTT GGT C (SEQ. ID. NO: 60)

12B5 Heavy chain variable domain

5 primer

CAT TCT ACA AAC GCG TAC GCT CAG GTG CAG CTG GTG CAG (SEQ. ID. NO: 62)

15 primer

GTA AAT GTA TGG GCC CTT GGT GGA GGA GGC ACT CGA GAC GGT GAC (SEQ. ID. NO:

63)

12D5 Heavy chain variable domain

5 primer

CAT TCT ACA AAC GCG TAC GCT CAG GTG CAG CTG GTG GAG (SEQ. ID. NO: 64)

3 primer

GTA AAT GTA TGG GCC CTT GGT GGA GGA GGC ACT CGA GAC GGT GAC (SEQ. ID. NO:

63)

10D10 Heavy chain variable domain

5 primer

CAT TCT ACA AAC GCG TAC GCT GAC GTG CAG CTG GTG CAG (SEQ. ID. NO: 65)

3 primer

GTA AAT GTA TGG GCC CTT GGT GGC GGC TGA GGA GAC GGT GAC (SEQ. ID. NO: 66)

12E10 Heavy chain variable domain

30 primer

CAT TCT ACA AAC GCG TAC GCT CAG GTG CAG CTG CAG CAG (SEQ. ID. NO: 67)

3 primer

GTA AAT GTA TGG GCC CTT GGT GGA GGA GGC ACT CGA GAC GGT GAC (SEQ. ID. NO:

63)

35 10F6 Heavy chain variable domain

5 primer

CAT TCT ACA AAC GCG TAC GCT CAG GTG CAG CTG CAG GAG (SEQ. ID. NO: 68)

3 primer

GTA AAT GTA TGG GCC CTT GGT GGA GGC TGA AGA GAC GGT AAC (SEQ. ID. NO: 69)

PCR reactions were carried out using 100 ng of plasmid DNA containing the scFv, 0.5  $\mu$ M of the appropriate 5 and 3 primer, 200  $\mu$ M each dNTP, 10 mM KCl, 6 mM(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM TRIS-HCl, pH 8.0, 2 mM MgCl<sub>2</sub>, 1% Triton X-100, 100  $\mu$ M BSA and 2.5 units of Pfu DNA polymerase (Stratagene). Amplification was for 30 cycles of: 30 sec at 95 °C, 30 sec at 55°C, 30 sec at 72°C. After digestion with the appropriate 5 restriction enzymes, the reaction products were separated by agarose gel electrophoresis and the approximately 350 bp band was isolated using a Gene Clean II kit (BIO 101, Vista, CA). The fragments for the light chain variable regions were ligated into the vectors previously digested with Sfi I and Rsr II for the kappa isotypes, or Sfi I and Msc I for the lambda isotypes, and transformed into *E. coli* DH5a. Desired recombinants were identified using restriction enzyme analysis and sequenced to confirm the presence of the desired fragments. 10 The heavy chain variable domains were then cloned similarly into the plasmids containing the light chains using the restriction enzymes Mlu I and Apa I, and the final constructions were again checked by DNA sequencing.

(k) Construction of scFv with gD tags.

For increased and regulated expression in high density fermentation tanks, the Sfi I to Not I fragments 15 of the scFv forms of p12B5, p12D5, p10F6, and p12E10 were subcloned into a derivative of pAK19 containing the *phoA* promoter and stII signal sequence rather than the *lacZ* promoter and hybrid signal sequence of the original library. For ease of purification, a DNA fragment coding for 12 amino acids (met-ala-asp-pro-asn-arg- 20 phe-arg-gly-lys-asp-leu) (SEQ. ID. NO: 70) derived from herpes simplex virus type 1 glycoprotein D (Lasky and Dowbenko *DNA* (N.Y.) 3:23-29 (1984.)) was synthesized and inserted at the 3 end of the V<sub>L</sub> domain in place of the (his)<sub>6</sub> and *c-myc* epitope originally present in the C.A.T. library clones.

(l) Expression in *E. coli*

Plasmids containing genes for scFv-gD, Fab' or Fab'2 molecules were expressed in *E. coli* strain 33B6 (W3110 *DshuA phoADE15 deoC2 ilvG2096(val<sup>R</sup>) degP41(DPstI-Kan<sup>R</sup>) D(argF-lac)169 IN(rrnD-rrnE)1*) 25 grown for approximately 40 hr at 30°C in an aerated 10-liter fermentor as described previously (Carter *et al* *Bio/Technology* 10:163-167(1992.)).

Example 3

Cloning and expression of full length human antibody derivatives of 12B5, 12D5, and 12E10.

For expression of full length antibodies in mammalian cells, the heavy chain variable domains were 30 subcloned from the Fab constructs into a derivative of expression vector pRK (Suva *et al.*, *Science* 237:893-896 (1987)) which contains the human IgG1 CH1, CH2, and CH3 domains and a human antibody signal sequence (Carter *et al.*, *Proc. Natl. Acad. Sci. USA.* 89:4285-4289 (1992)). The light chain was cloned into a separate pRK plasmid. The light and heavy chain expression vectors were cotransfected into adenovirus-transformed human embryonic kidney cell line 293 by a high-efficiency procedure (Gorman *et al*, *DNA Protein Eng. Technol.* 2:3-10 (1990)). Harvested conditioned media was shown to contain anti-mpl antibody by 35 ELISA.

For production of a more stable cell line and high-level antibody production, the light and heavy chains were moved into the SVI.DI expression vector previously described (Lucas *et al.*, *Nucleic Acids Res.* 24: 1774-1779 (1996)). This vector contains the mouse DHFR cDNA in the intron of the expression vector pRK and allows for amplification of expression by selection in methotrexate. The light chain is cloned into the same

plasmid with expression driven by a second SV40 promoter/ enhancer. The plasmid was linearized and transfected into CHO cells using lipofectamine (Gibco-BRL) following manufacturer's instructions. Seven to ten days after transfer to selective medium, clones were isolated into 96 well plates for later study, or pooled and expanded for culture in roller bottles.

5        Conditioned media for purification of the antibodies was generated in roller bottles. Cells were seeded into the roller bottles at an initial cell density of  $2 \times 10^7$  cells in 200 ml rich medium (DMEM: Ham's F12 (1:1) supplemented with 5% fetal bovine serum. At approximately 80% confluence, the media was replaced with serum-free PS-24 production medium supplemented with insulin (10  $\mu$ g/ml), transferrin (10  $\mu$ g/ml), trace elements and lipid alcohol. Conditioned media was harvested after 10 days.

10

Example 4Purification Of Agonist Antibodies(a) Purification of scFv with gD tag

Frozen cell paste was resuspended at 1 gm/ml TE (25 mM TRIS, 1 mM EDTA, pH 7.4) and gently agitated 18 hr on ice. Cell debris was removed by centrifugation at 10,000 x g for 30 min. The supernatant was 15 loaded onto an affinity column (2.5 x 9.0 cm) consisting of an anti-gD monoclonal antibody 5B6 (Paborsky, L. R. *et al.*, *Protein Eng.* 3: 547-553 (1990)) coupled to CNBr SEPHAROSE which had been equilibrated with PBS. The column was washed 18 hr with PBS, and then washed with PBS containing 1 M NaCl until the absorbance of the column effluent was equivalent to baseline. All steps were done at 4°C at a linear flow rate of 25 cm/hr. Elution was performed with 0.1 M acetic acid, 0.5 M NaCl, pH 2.9. Column fractions were 20 monitored by absorbance at 280 nm and peak fractions pooled, neutralized with 1.0 M TRIS, pH 8.0, dialyzed against PBS, and sterile filtered. The resultant protein preparations were analyzed by non-reducing SDS-PAGE.

(b) Purification of Fab' molecules

For purification of Fab' molecules, 5 g of frozen cell paste was resuspended in 5 ml of TE (25 mM TRIS, 1 mM EDTA, pH 7.4) and gently stirred 18 hr on ice. The pH of the shockate was adjusted to 5.6 with 2 M HCl and the precipitate and cell debris removed by centrifugation at 10,000 x g for 30 min. The supernatant was loaded onto a 1 ml BAKERBOND ABx column (0.5 x 5.0 cm) (J. T. Baker, Phillipsburg, NJ) pre-equilibrated with 20 mM MES, pH 5.5. After washing with 20 mM MES to baseline, the Fab' was eluted using a 10 ml linear gradient from 0 to 100% of 20 mM NaOAc, 0.5 M  $(\text{NH}_4)_2\text{SO}_4$ , pH 7.2, with a flow rate of 153 cm/hr. Fractions containing Fab' were pooled, and buffer exchanged into PBS.

(c) Purification of Fab'2 molecules

Frozen cell paste (100 gm) was thawed into 10 volumes of 25 mM TRIS, 5 mM EDTA, 1 mM NaN3, pH 7.4 and disrupted by three passages through a microfluidizer (TECH-MAR). PMSF was added to 1 mM and the cell debris removed by centrifugation at 10,000 x g for 30 min. The supernatant was filtered sequentially 35 through a 0.45 $\mu$ m, and a 0.2  $\mu$ m SUPORCAP filter (Gelman), and loaded onto a 50 ml SEPHAROSE-fast-flow Protein-G column (Pharmacia) pre-equilibrated with PBS. After washing to baseline with PBS, Fab'2 was eluted with 0.1 M glycine ethyl ester, pH 2.3, into tubes with contained 1/10 volume of 1 M TRIS, pH 8.0. Fractions containing Fab'2 were pooled and concentrated by Ultrasette with a 30 kilodalton molecular weight cut off, and buffer exchanged into 20 mM NaOAc, 0.01% octylglucoside, pH 5.5. This material was loaded

onto a 30 ml S-SEPHAROSE column (Pharmacia) pre-equilibrated with 20 mM NaOAc, washed to baseline with 20 mM NaOAc, pH 5.5, and eluted with a linear gradient of 0-1 M NaCl in 25mM NaOAc over 10 column volumes. Fractions containing Fab'2 were pooled and buffer exchanged to PBS.

(d) Purification of full length antibodies from transfected CHO cell supernatants.

5 Conditioned medium harvested from roller bottles was loaded onto a 5 ml Protein-A SEPHAROSE column (1.0 x 5.0 cm) pre-equilibrated with PBS, washed with PBS, and then washed to baseline with PBS containing 1 M NaCl. Antibody was eluted with 0.1 M HOAc, 0.5 N NaCl, pH 2.9, neutralized with 1 M TRIS, and buffer exchanged to PBS.

10 A summary of agonist antibody activities for several antibodies and fragments thereof is shown in Table 4 below.

Table 4  
Summary of Mpl Agonist Antibody Activities

Antibody	Hu3 Proliferation (ED50)	KIRA (ED50)	Hu3 Binding (IC50)	Mpl/TPO ELISA (IC50)	Platelets (IC50)	MK Assay
<u>12B5</u> scFv	20 pM	1 nM	10 nM	17nM	100 nM	++
Fab	>1μM	3 nM	900 nM	none	>1μM	-
Fab'2	5 pM	1 nM	5 nM	1 nM	300 nM	+
IgG	30 pM	400 pM	10 nM	152 pM	300 nM	-
<u>12E10</u> scFv	5 pM	60 pM	5 nM	1.6 nM	5 nM	
Fab	>1μM	>1μM	500 nM	180 nM	>1μM	
Fab'2	>1μM	160 pM	10 nM	640 nM	500 nM	
IgG	>1μM	480 pM	50 nM	450 pM	500 nM	
<u>12D5</u> scFv	1.2 nM	280 pM	10 nM	24 nM	>1μM	
Fab	>1μM	4 nM	500 nM	1μM	>1μM	
Fab'2	4.8 pM	600 pM	4 nM	1 nM	100 nM	+
IgG	>1μM	3 nM	10 nM	450 pM	500 nM	

Example 5

15 In another embodiment, the invention provides a method of selecting an antibody which binds to and dimerizes a receptor protein. In this method, a library of antibodies is panned using a receptor protein having two protein subunits as the binding target. The library is panned as described above for mpl agonist antibodies. Preferably, the antibodies are human and more preferably monoclonal. The library is conveniently a library of single chain antibodies, preferably displayed on the surface of phage. The display of proteins, including 20 antibodies, on the surface of phage is well known in the art as discussed above and these known methods may be used in this invention. Antibody libraries are also commercially available, for example, from Cambridge Antibody Technologies (CAT), Cambridge, UK. Preferably, the antibody selected by the method of the invention activates the receptor by dimerizing the receptor and thereby achieves an effector result similar to the effector result generated when the natural endogenous ligand for the receptor binds the receptor.

25 The method of the invention can be used to find agonist antibodies to any receptor having two components which is known and /or can be cloned. It is not necessary to know the primary, secondary or

tertiary structure of the receptor protein, although this information is useful for cloning, etc., since the method of the invention allows selection of antibodies which will bind any displayed receptor which is activated by dimerization. Many known receptor proteins are activated by dimerization and any of these known receptors may be used in the invention. Suitable receptors include tyrosine kinase receptors and hematopoietic receptors 5 that lack kinase activity.

Activation of a receptor such as a tyrosine kinase receptor by a scFv is an unexpected result. Current understanding of receptor activation argues that for many classes of receptors, including tyrosine kinase receptors and hematopoietic cytokine receptors that lack intrinsic tyrosine kinase activity (but associate with intracellular kinases), it is a dimerization event mediated by a ligand that is the key event in receptor activation. 10 This view is supported by crystal structures of receptor ligand complexes as well as the demonstrated agonist ability of certain monoclonal antibodies (but not the Fab' fragments of these antibodies). A single chain antibody would not, therefore, be expected to be able to cause receptor dimerization and activation.

MuSK is a recently identified tyrosine kinase localized to the postsynaptic surface of the neuromuscular junction. (Valenzuela *et. al.* 1995, *Neuron* 15 573-584.) Mice made deficient in MuSK fail 15 to form neuromuscular junctions (Dechiara *et. al.* 1996, *Cell* 85 501-512.), a phenotype highly similar to that observed in mice lacking the nerve derived signaling molecule agrin (Gautam *et. al.*, 1996, *Cell* 85 525-535). The likely involvement of MuSK in agrin signaling is strengthened by the observations that agrin induces the rapid tyrosine phosphorylation of MuSK and that labeled agrin can be chemically crosslinked to MuSK (Glass *et. al.*, 1996, *Cell* 85 513-523.).

Formation of the neuromuscular junction is achieved through a process that includes the differentiation 20 of membrane on the muscle fiber proximal to the neuron terminus and changes in gene expression within the nuclei proximal to this junction (reviewed by Bowe *et. al.*, 1995, *Annu-Rev-Neurosci.* 18 443-462 and Kleiman *et. al.*, 1996, *Cell* 85 461-464.). A striking feature of this complex process is the redistribution and concentration of AChRs within the myotube membrane. Agrin is able to induce this clustering of AChRs as 25 well as changes in the extracellular matrix and cytoskeletal components of the synaptic apparatus (Bowe *et. al.*, *supra*; Godfrey *et. al.*, 1984, *J. Cell Biol.* 99 615-627; Nitkin *et. al.*, 1987, *J. Cell Biol.* 105 2471-2478). Agrin is a secreted protein with a core molecular weight of ~200 kDa that contains several copies of EGF repeats, laminin-like globular domains and sequences that resemble protease inhibitors. It is released by motor neuron terminals and maintained within the basal lamina of the synaptic cleft. While agrin apparently does not 30 to bind MuSK with high affinity (Glass *et. al.*, *supra*), it has been reported to interact with other molecules present at the neuromuscular junction, most notably alpha-dystroglycan (O'Toole *et. al.*, 1996, *Natl. Acad. Sci. USA* 93 7369-7374) thereby complicating the analysis of MuSK's role in the signaling events initiated by agrin.

Antigen specific scFv, identified by panning a diverse library of scFv expressed, for example, on M13 phage provide a source of molecules capable of mediating specific therapeutic activities, and offer a rapid new 35 approach to study the function of novel or recently identified molecules such as MuSK. scFv are identified below that mediate receptor activation and that direct MuSK activation induces changes in AChR distribution and tyrosine phosphorylation similar to that observed with agrin.

The induction of AChR clustering and tyrosine phosphorylation by scFv antibodies provides direct evidence to support conclusions drawn from studies of knockout mice deficient in MuSK indicating this

recently discovered tyrosine kinase acts to induce key events in the formation of the neuromuscular junction. As a potential signal transducer of agrin, it is noteworthy that MuSK does not display high affinity binding to agrin, leading to speculation that there must be an additional agrin binding component(s) involved in mediating the agrin signal. The molecular nature of this component is unknown. It is interesting that it is possible to 5 induce the receptor clustering, the hallmark activity of agrin, with an agent directed specifically to MuSK.

The marked upregulation of MuSK expression in muscle following denervation or muscle immobilization as well as the chromosomal localization of MuSK within a region associated with fukiyama muscular dystrophy point to an important role for this molecule in regulation of the neuromuscular junction (Valenzuela *et.al.*, *supra*) and indicates the possibility that therapeutic benefit is possible through the controlled 10 regulation of MuSK activity. As agrin is expressed not only at the neuromuscular junction, but in a wide variety of peripheral and central neurons (Bowe *et. al.*, *supra*; Rupp *et. al.*, 1991, *Neuron* 6 811-823; Tsim *et. al.*, 1992, *Neuron* 8 677-689) it may not be an optimal candidate molecule through which to manipulate MuSK function as exogenously introduced agrin derivatives might elicit consequences not restricted to the neuromuscular junction. Thus, in comparison, the ability to obtain direct activation of MuSK through scFv 15 offers an attractive alternative. Each of the scFv that were tested displayed affinity for MuSK in the nM range demonstrating the utility of phage displayed scFv libraries as a rich source of high affinity and highly specific molecules.

The antibodies of the invention are, therefore, useful in assaying the upregulation of MuSK receptors in sample tissues to determine the degree of neuromuscular damage associated with this upregulation. The 20 antibodies are also useful for activating the MuSK receptor and inducing AChR clustering at neuromuscular junctions as a direct result of the agonist properties of these antibodies. Administration of the antibodies to a person suffering from denervation or muscle immobilization, e.g. muscular dystrophy, provides a method of improving the function of the neuromuscular junctions in these people.

To prepare scFv having agonist activity, antibodies were selected which induce a proliferative 25 response in a factor dependent cell line through a chimeric MuSK-Mpl receptor comprised of the extracellular domain of MuSK and the intracellular domain of the hematopoietic cytokine receptor c-Mpl (the receptor for thrombopoietin, TPO). Activation of c-Mpl is believed to require homodimerization, as is the case for the growth hormone receptor, the erythropoietin receptor and other related receptors of this class (Carter *et. al.*, 1996, *Annu-Rev-Physiol* 58 187-207; Gurney *et. al.*, 1995, *Proc .Natl. Acad. Sci. USA* 92 5292-5296). 30 Ba/F3 cells expressing MuSK-Mpl were starved of IL-3 and exposed to a range of concentrations of each scFv expressed as soluble protein. Surprisingly, 4 of the 21 scFv were able to induce a robust proliferative response in the MuSK-Mpl expressing cells (Fig. 11). This activity was observed at nM concentrations of scFv. The scFv were without effect on the parental, untransfected Ba/F3. Agonist activity was also present among those IgG that were derived from agonist scFv but was not noted among IgG derived from non-agonist scFv. Soluble 35 agrin c-terminal domain (c-agrin) was without effect supporting previous observations that agrin does not bind MuSK directly. The c-terminal domain of agrin is known to contain the AChR clustering activity of agrin and is essential for neuromuscular junction formation (Ruegg *et al.*, 1992, *Neuron* 8 691-699; Tsim *et. al.*, *supra*). The EC<sub>50</sub> for the ability to induce proliferation was 5 nM for the most active agonist clone when expressed as either scFv or IgG. The affinity of these scFv and IgG for MuSK was determined using BIACore<sup>TM</sup> analysis.

The agonist scFv and several non-agonist scFv each displayed affinity for MuSK within the range of 5-25 nM. In contrast, the affinities of the IgG for MuSK were 10-30 pM. See Table 5 below.

Table 5

clone #	Agonist	$k_d$	$k_a$	Affinity
musk #2-scFv	+	$3.34 \times 10^{-3}$	$8.78 \times 10^5$	3.8 nM
musk #3-scFv	-	$2.39 \times 10^{-3}$	$1.05 \times 10^5$	23 nM
musk #4-scFv	+	$1.57 \times 10^{-3}$	$1.84 \times 10^5$	8.5 nM
musk #5-scFv	-	$2.49 \times 10^{-3}$	$5.29 \times 10^5$	4.7 nM
musk #6-scFv	-	$4.95 \times 10^{-3}$	$1.05 \times 10^5$	4.7 nM
musk #13-scFv	+	$2.32 \times 10^{-3}$	$4.53 \times 10^5$	5.1 nM
musk #22-scFv	+	$6.09 \times 10^{-3}$	$1.27 \times 10^5$	4.8 nM
musk #13-IgG	+	$1.01 \times 10^{-5}$	$8.05 \times 10^5$	12.5 pM
musk #22-IgG	+	$4.86 \times 10^{-5}$	$1.65 \times 10^6$	29.5 pM

5 To probe this agonist activity further, scFv were examined for the ability to induce tyrosine phosphorylation of full length MuSK tyrosine kinase. The murine myoblastic cell line C2C12 was cultured under conditions that promote myotube differentiation and subsequently exposed to scFv, IgG or c-agrin. In correspondence with previous data (Glass *et. al.*, *supra*), c-agrin was able to induce MuSK tyrosine phosphorylation. The agonist scFv and IgG were also found to rapidly induce tyrosine phosphorylation of 10 MuSK as determined by western blot analysis with anti-phosphotyrosine antibody whereas other scFv and non-agonist anti-MuSK IgG were without effect.

15 The ability of the scFv MuSK agonists to induce AChR clustering in cultured C2C12 myotubes was examined. Following stimulation, the cells were fixed and the distribution of cell surface AChR was revealed with rhodamine labeled bungarotoxin. In undifferentiated myoblasts, AChR were dispersed and unfocused in the presence of c-agrin, scFv, or IgG. In contrast, upon myotube differentiation, c-agrin and agonist scFv and IgG induced marked aggregation of AChR into large and intensely stained clusters. Non-agonist scFv and non-agonist IgG directed against MuSK or an irrelevant antigen were without effect. An additional consequence of agrin action, tyrosine phosphorylation of subunits of the AChR was also examined utilizing an antisera that 20 recognizes the  $\alpha$  and  $\beta$  chains of the receptor. Tyrosine phosphorylation levels of both the  $\alpha$  and  $\beta$  chains were markedly induced by c-agrin as well as the agonist scFv and agonist IgG but were unaffected by control scFv and IgG.

Variants of the MuSK agonist antibodies of the invention may be prepared as described above for thrombopoietic antibodies.

25 Construction of expression vectors. Coding sequence for murine MuSK was obtained by PCR amplification. MuSK-Fc was prepared by fusion of the extracellular domain of MuSK (a.a. 1-492) in frame with the Fc region of human IgG1 in the eukaryotic expression vector pRK5tkNEO. MuSK-Fc was transiently expressed in 293 cells and purified over a protein G column. A chimeric receptor, MuSK-Mpl, comprised of the extracellular domain of MuSK (amino acids 1-492) and the transmembrane and intracellular domain of the human c-Mpl receptor (amino acids 491-635) was prepared by sequential PCR and cloned into pRK5tkNEO. 30 Stable cell lines expressing the chimeric receptor were obtained by electroporation (5 million cells, 250 volts, 960  $\mu$ F) of linearized vector (20  $\mu$ g) into Ba/F3 cells followed by selection for neomycin resistance with 2

mg/ml G418. Full length MuSK in pRK5tkNEO was transfected into 293 cells and stable transformants were obtained following two weeks of G418 selection (400 µg/ml). The sequence of the DNA constructs were confirmed by DNA sequencing. Expression of MuSK was assessed by flow cytometry analysis as described below. Ba/F3 cells were maintained in RPMI 1640 media supplemented with 10% fetal calf serum and 5% conditioned media from WEHI-3B cells as source of IL-3. C-agrin (amino acids 1137-1949 of the rat agrin (Ag +8 active splice form (Ferns *et al*, 1993, *Neuron* 11 491-502.)) was expressed by transient transfection from 293 cells in serum free media with an expression vector, pRK-gD-c-Agrin, as a fusion protein with the gD signal sequence and epitope tag and a genenase cleavage site (MGGAAARLGAVILFVV IVGLHGVRGKYALADASLKMADPNRFRGKDLPVLDQLLEGGAAHYALLPG) (SEQ ID NO. 71) fused to the N-terminus.

**Isolation of scFv and IgG** MuSK-Fc immunoadhesin was coated on Maxisorp tubes (Nunc) at 10 µg/ml. A library of human scFv (Cambridge Antibody Technology, England) was panned through two rounds of enrichment essentially as described (Griffiths *et al*, 199, *EMBO-J* 12 725-734). The specificity of individual clones was assessed first by elisa (Griffiths *et al*, *supra*) using MuSK-Fc and a control immunoadhesin (CD4-Fc). Positive clones were screened by PCR and "fingerprinted" by BstN1 digestion (Clackson *et al*, 1991, *Nature* 352 642-648.). Examples of clones with unique patterns were sequenced and subjected to FACS analysis with cells expressing or not expressing MuSK. For FACS analysis, cells ( $10^5$ ) were incubated for 60 minutes at 4 C in 200 µl 2% FBS/PBS (fetal bovine serum/phosphate buffered saline) with  $10^{10}$  phage that were first blocked by incubation in 30 µl 10% FBS/PBS. Cells were then washed with 2% FBS/PBS, stained with anti-M13 antibody (Pharmacia, Piscataway NJ) and R-phycoerytherin-conjugated donkey anti-sheep antibody (Jackson Immunoresearch, West Grove PA), and analyzed by FACS analysis. ScFv were expressed in bacteria as epitope tagged proteins containing a *c-myc* tag sequence recognized by monoclonal antibody 9E10 (Griffiths *et al*, *supra*) and a polyhistidine tail (his<sub>6</sub>) and were purified over Ni-NTA column with imidazole elution as recommended by manufacturer (Qiagen). For expression of clones as IgG the sequences encoding the V<sub>H</sub> and V<sub>L</sub> regions of the scFv were introduced by PCR into mammalian expression vector pIgG-kappa which was designed to enable the expression of fully human light and heavy chains of kappa type IgG. Expression vectors for the individual clones were transfected into CHO cells and IgG were harvested from conditioned serum free media and purified over a protein A column.

**Proliferation assays.** Cells were cultured in the absence of IL-3 for twenty-two hours (in RPMI supplemented with 10% FBS). Cells were then washed twice with RPMI and plated in 96 well dishes at 50,000 cells per well in 0.2 ml of 7.5% FBS RPMI supplemented with the indicated concentrations of scFv or IgG. Each concentration was tested in duplicate. After an incubation of sixteen hours, 1 µCi of [<sup>3</sup>H]-thymidine was added per well and incubation was continued for an additional six hours. Incorporation of [<sup>3</sup>H]-thymidine was measured with a Top Count Counter (Packard Instruments, CA).

**AChR clustering assay.** C2C12 were maintained in 10% FBS in high glucose DMEM at subfluency. For AChR clustering assays C2C12 were seeded on glass slides coated with fibronectin and poly-lysine and myotube differentiation was induced by 48 hour incubation in 2% horse serum high glucose DMEM. scFv or c-agrin were added to the culture medium and incubated overnight (16 hours). Cells were then washed with PBS

and fixed in 4% paraformaldehyde. Rhodamine conjugated bungarotoxin (Molecular Probes, Eugene OR) was used to reveal the localization of AChRs as described (Ferns *et al, supra*).

Binding affinity analysis. Protein interaction analysis using BIACoreTM instruments was performed as described (Mark *et al, 1996, J. Biol. Chem. 271* 9785-9789). Briefly, research grade CM5 sensor chips were activated by injection of 20  $\mu$ l of 1:1 N-Ethyl-N'-(3-dimethylaminopropyl)carboiimide hydrochloride and N-hydroxysuccinimide at 5  $\mu$ l /min flow rate. 20  $\mu$ l of MuSK-Fc at 20  $\mu$ g/min in 10 mM sodium acetate, pH 5.0 was injected over the sensor chip, followed by 30  $\mu$ l of ethanolamine. scFv or IgG were purified and concentrations determined by Pearce BCA kit. Thirty  $\mu$ l protein samples in PBS with 0.05% Tween 20 were injected at a flow rate of 10  $\mu$ l min by the Kinject method. Proteins were allowed to dissociate for 20 min in a flow of PBS with 0.05% Tween 20. Sensorgrams were analyzed with BIAevaluation 2.1 software from Pharmacia Biosensor AB. Apparent dissociation rate constants ( $k_d$ ) and association rate constants ( $k_a$ ) were obtained by evaluating the sensorgram with A + B = AB type I fitting. Equilibrium dissociation constant  $K_d$  was calculated as  $k_d/k_a$ .

Immunoprecipitation and Western Blot Analysis. C2C12 were maintained in 10% FBS high glucose DMEM and induced to differentiate by 72 hour incubation in 2% horse serum. Cells were then stimulated by addition of c-agrin, scFv or IgG for the time indicated in the figures. ScFv and IgG were used at 50 nM. The c-agrin containing conditioned media was used at level that provided maximal tyrosine phosphorylation. Cell extracts were prepared as described (Gurney *et al, supra*). Extracts were incubated for 60 minutes at 4°C with 30  $\mu$ l agarose conjugated antiphosphotyrosine monoclonal antibody 4G10 (UBI inc., Lake Placid NY) or 1  $\mu$ g of anti-MuSK IgG #13 followed by 30  $\mu$ l protein A sepharose beads. Western blot analysis with antiphosphotyrosine antibody 4G10 or anti AChR antibody (Affinity Bioreagents, Golden CO) was performed as recommended by the manufacturer and revealed with HRP conjugated secondary antibody and ECL (Amersham).

MuSK scFv are readily observed as dimers when resolved by nondenaturing gel electrophoresis. Additionally, the abundance of dimeric species may be significantly altered in the local context of scFv bound to receptor on the cell surface. Alternatively, screening an scFv phage library with a divalent antigen, in this case MuSK-Fc, allows direct selection of scFv that bind to and facilitate the formation of a receptor dimer.

While the invention has necessarily been described in conjunction with preferred embodiments and specific working examples, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and alterations to the subject matter set forth herein, without departing from the spirit and scope thereof. Hence, the invention can be practiced in ways other than those specifically described herein. It is therefore intended that the protection granted by letters patent hereon be limited only by the appended claims and equivalents thereof.

All references cited herein are hereby expressly incorporated by reference.

#### Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia, USA (ATCC):

	<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
40	pMpl.12B5.scFv.his	_____	August 18, 1998

Missing Dep. #'

	pMpl.12D5.scFv.his	_____	August 18, 1998
	pMpl.12E10.scFv.his	_____	August 18, 1998
	pMpl.10D10.scFv.his	_____	August 18, 1998
5	pMpl.10F6.scFv.his	_____	August 18, 1998
	pMpl.5E5.scFv.his	_____	August 18, 1998

10 This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 15 886 OG 638).

20 The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

25 The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.